from a test sample is present, analyte-specific antibody is diverted away from the second member position of the tridentate by the free analyte, thereby decreasing modulation and increasing complex formation, as is shown in Figure 1C. This results in a positive correlation of increased signal, or complex formation, with increasing concentrations of free hapten.

In addition to the suggested biotin-theophylline-DNP tridentate, other tridentates, including biotin-theophylline-biotin, will work equally well in a NIIA for theophylline or theophylline-amine where theophylline is also the second (modulating) member of the tridentate. Examples of other drugs which can also be assayed effectively in a competitively modulated assay such as a NIIA, using the tridentate of the present invention, include therapeutic drugs such as digoxin, disopyramide, lidocaine, procainamide, propanolol, quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, and drugs of abuse such as morphine, codeine, and heroin, and their matabolites. Examples of other haptens which can be assayed using the tridentate conjugate include DNP, 1-substituted-4-hydroxy-2-nitro-benzene, and 4-substituted-2-nitro-trialkylanilinium salts.

Unlike prior art developer antigens, the tridentate is a stable, chemically defined organic compound which enjoys prolonged shelf life, and does not require the expensive purification and characterization procedures inherent with the prior art conjugates. The tridentate of the present invention also yields improved sensitivity, due to the fact that complexing is based on

the competition of only one moiety on the tridentate, namely the second (modulating) member with free analyte, rather than the competition of the plurality of hapten moieties conjugated to the typical prior art developer antigen. The prior art problem of nonproductive binding is also eliminated.

B. Proximity Assays

This same universal tridentate can generally be applied to other types of competitively modulated assays, including those using proximity labels. These assays are particularly effective in detecting the same group of analytes previously mentioned. In these assays, the second tridentate member again acts as the modulator. One proximity label is attached to the macromolecular specific binding partner for the first member of the tridentate conjugate. The other proximity label is attached to the macromolecular specific binding partner for the third member of the tridentate conjugate. Where the two macromolecules simultaneously bind to the first and third tridentate members, a measurable reaction takes place between the two proximity labels. Where specific binding partner to the second tridentate member; i.e., analyte-specific antibody, binds to the modulating second member of the tridentate, at least one of the labeled macromolecules will be sterically inhibited from simultaneously binding to either the first or third tridentate member, thus reducing signal.

The section of the spacer moiety connecting the first and third tridentate members of the universal tridentate must again be of sufficient length to allow the simultaneous binding of the labeled macromolecules. However, at the same time, the length of this section of the spacer must be sufficiently short to bring the labels

into close enough proximity to obtain a measurable reaction. The length required to achieve the required proximity will vary to some degree with the particular type of proximity assay chosen, but is, in general, relatively long. For example, energy transfer between proximity labels can ordinarily occur at lengths up to 70 Å.

In an enzyme channeling assay, the first enzyme is preferably attached to the specific binding partner of either the first or third tridentate member, with the second enzyme being attached to the corresponding specific binding partner of the remaining first or third member. Good enzyme pairs for use in an enzyme channeling assay include glucose oxidase and peroxidase, and hexokinase and glucose-6-phosphate dehydrogenase. The second tridentate member is identical or analogous to the analyte of interest, and competes with free analyte from a test sample for a limited amount of antibody to the analyte of interest. In the absence of binding by the analyte-specific antibody to the second tridentate member, macromolecules carrying the first and second enzymes will be able to simultaneously bind to the first and third tridentate members such that the product of the first enzyme can be converted by the second enzyme before the product escapes into bulk solution.

Where, however, little or no free analyte is contributed by the test sample, analyte-specific antibody will be able to bind to the second tridentate member, sterically blocking the binding of the respective enzyme-labeled macromolecules to the first and/or third tridentate members, thereby modulating enzyme channeling; i.e., reducing signal. The greater the amount of analyte from a test sample, the less antibody will be able to bind to the tridentate. Modulation will decrease, and

enzyme channeling will increase, as will the amount of signal generated.

Similarly, in an energy transfer assay, the donor label is preferably attached to the specific binding partner of either the first or third tridentate member, with the acceptor label being attached to the corresponding specific binding partner of the remaining first or third member. As with the NIIA and enzyme channeling methods, the second tridentate member is identical or analogous to the analyte of interest. The second tridentate member competes with free analyte from a test sample for a limited amount of antibody to the analyte of interest. The greater the amount of antigen contributed by a test sample, the more analyte-specific antibody will be diverted from its modulating position wherein it is bound to the second tridentate member. Lesser amounts of analyte result in less energy transfer and, therefore, less signal.

Several donor-acceptor pairs are available for use in energy transfer assays employing the tridentate of the present invention. The donor label should generally be a compound which absorbs external energy and emits light energy. Examples of good donor labels include fluorescent compounds, scintillation dyes, and chemiluminescent compounds such as isoluminol and acridin ester. Acceptor labels are usually fluorescent compounds which can absorb the energy emitted by the donor and, in turn, emit fluorescence at a wavelength longer than that of the donor's emitted light energy. Preferably, acceptors should have a good fluorescence efficiency. Good acceptor labels include fluorescein, rhodamine, fluorescent lanthanide chelates, and fluorescent tin or zinc derivatives of protoporphyrins. Rhodamine is a particularly good acceptor label, because it is capable

of absorbing energy emitted by a donor over a wide spectrum of wavelengths. Examples of good donor:acceptor pairs are: isoluminol:fluorescein, acridin ester: fluorescein, and fluorescein: rhodamine.

In yet another preferred application of the steric hindrance embodiment of the invention, a tridentate other than the universal tridentate is utilized in competitively modulated assays employing proximity labels. In these assays, a tridentate is used wherein only two of the tridentate members are small molecule ligands. One of these small molecule ligand members is preferably the second, or modulating, member of the tridentate which typically competes with free analyte for a limited amount of analyte-specific antibody. The other small molecule ligand member binds to its macromolecular specific binding partner which has been conjugated to a first proximity label. Where analyte-specific antibody is bound to the second member position, the labeled macromolecule is sterically inhibited from binding to the same tridentate.

In the absence of modulation by the second member, the first proximity label is preferably brought into proximity with a second proximity label in one of two ways: (1) the second proximity label is conjugated directly to the tridentate as a tridentate member; or, (2) a plurality of second proximity labels is attached to a large macromolecule or solid support, which is conjugated directly to the tridentate as a tridentate member. In the latter instance, particularly where one of the tridentate members is a solid support, a multiplicity of tridentates may share the same solid support member, as is shown in Fig. 11. The extent to which the specific binding partner containing the first proximity label is able to bind to the tridentate

controls the extent of signal generated. Free analyte from a test sample increases this signal by diverting analyte-specific antibody away from its modulating position on the tridentate.

The tridentate of the present invention provides the same general advantages in proximity assays as in nephelometric assays. The defined chemical nature of the synthesized organic tridentate alleviates many of the problems typically encountered in dealing with the naturally occurring proteinaceous substances which form the basis for most prior art reagents. The tridentate does not require the expensive isolation and characterization procedures of prior art reagents, and exhibits a much longer shelf life than its prior art counterparts. The same haptens and drugs may be conveniently assay using these methods as may antigens where antigen fragments can be effectively employed as the modulating tridentate member.

2. Targeted Labeling

Another embodiment of the present invention utilizes a tridentate which is designed to employ a different steric phenomenon which may be referred to as region-specific labeling, or targeted labeling. At least two of the members of this tridentate are small molecules. These two small molecule members are capable of binding to the same macromolecule, and at least one of these two small molecule members is a chemically reactive group. The third tridentate member may be a small molecule ligand. It may also be a small molecule other than a small molecule ligand, or even a macromolecule. The targeted labeling embodiment of the tridentate can be advantageously used where it is desired to bind a small molecule ligand or oth r third tridentate member to a

targeted site or sites on a particular macromolecule. This third tridentate member may act as a label, tracer, or reporter group. It may also act as a solid support such as where the third tridentate member is a macromolecule. Biotin, for example, may be used as a small molecule ligand label. Radioactive compounds such as I^{125} are a good example of small molecule tracers. Enzymes are good macromolecular labels.

In general principle, the binding of a first small molecule tridentate member restricts the binding of a chemically reactive second small molecule member to a particular region on the same macromolecule. The small molecule tridentate member responsible for locating and initially binding to the targeted site on the macromolecule may be either a chemically reactive group or a small molecule ligand and is referred to as the guiding tridentate member. The small molecule tridentate member whose binding is thus restricted to the vicinity or region on the same macromolecule to which the guiding member has bound is referred to as the reactive tridentate member. The reactive member must be a chemically reactive group. The third tridentate member may be conveniently referred to as the "intended label", even where the third member is, in fact, an intended solid support. Preferably, the length of the section of the spacer moiety connecting the guiding member and the reactive member will be relatively short, while that connecting the reactive member and the intended label will be relatively long.

The targeted labeling embodiment of the present invention is of particular use in the conjugation of intended labels to proteins such as proteoglycans, lipoproteins, enzymes, antibodies, and receptors. As mentioned, most proteins contain a polysaccharide or

sugar moiety at a location distant from the active sit of the macromolecule. Proteins containing such a polysaccharide or sugar moiety may be referred to as glycosylated proteins. Unlike the prior art methods, the tridentate is capable of targeted modification at the polysaccharide moiety of a protein without incurring the harsh reaction conditions and nonspecific binding that can denature the protein.

For example, where the tridentate is to be used to accomplish the targeted labeling of a protein, the tridentate members are preferably selected as follows: (1) the guiding member is a phenylboronic acid residue; (2) the reactive member is a nitrophenylazido residue; and, (3) the remaining tridentate member is the intended label, such as biotin. The substituted boronic acid group of the guiding member specifically seeks out and reacts with the cis-diol group of the sugar moiety of the protein to form a relatively weak boronic ester complex, or bond. This reaction is carried out in the dark. After elimination of the excess of the tridentate conjugate, the reaction mixture is then exposed to ultra-violet light, which activates the azide group of the reactive member to nitrene. The nitrene then inserts into any chemical bond within the vicinity of the previously bound guiding member. This insures a permanent bond away from the active site of the protein. Subsequent release of the guiding member, such as through hydrolysis of the boronic ester bond due to pH changes, does not impair the functional utility of the tridentate conjugate. The third member is attached to the protein through a permanent bond. The reaction is specific and the reaction conditions are mild.

Synthesis of the Tridentate Conjugate

1. Generally

The synthesis of the tridentate is relatively simple once the ultimate use of the tridentate is determined, and the tridentate members are selected. These determinations, in turn, dictate the size and configuration of the spacer moiety.

Where the tridentate is intended for use in a modulated assay, such as a NIIA, enzyme channeling, or energy transfer assay, the tridentate members will preferably be selected as suggested above. Where use in other modulated assay methods is intended, the tridentate members will be selected in a similar manner. In other words, the second tridentate member will preferably be identical or analogous to an analyte of interest, and at least one of the first and third members will preferably be a different ligand selected from small molecule ligand:specific binding partner pairs. The remaining member will ordinarily be another small molecule ligand, a proximity label, or a large macromolecule or solid support.

Where the targeted labeling embodiment is intended, such as in the specific modication of a macromolecule, the guiding member is selected to be either a small molecule ligand or a chemically reactive group capable of selectively binding to a targeted site or sites on a designated macromolecule. The reactive member is selected to be a chemically reactive group capable of permanently binding to a location on the same macromolecule proximate to the location at which the guiding member has bound. The third tridentate member is ordinarily selected to be a label, tracer, or reporter

group, which may be a small molecule, a small molecule ligand, or a macromolecule, or a solid support, which is typically a macromolecular substance.

The tridentate conjugate is generally built around a starting spacer moiety, with each of the tridentate members being linked to each other through attachment onto the spacer moiety. There are many methods known in the art for linking together members of a bifunctional conjugate through spacer moieties. See, for example, U.S. Patent No. 4,134,792, U.S. Patent No. 4,238,565, and Green, N.M., Konieczny, L., Toms, E.J., and Valentine, R.C., The Use of Bifunctional Biotinyl Compounds to Determine the Arrangement of subunits in Avidin, Biochem. J., 125, 781-791 (1971). These methods generally employ typical condensation, addition, and substitution reactions between the reactive groups of two different organic compounds which may or may not have been activated prior to conjugation. The same or similar methods may ordinarily be applied to attach the tridentate members to the selected spacer moiety as is more particularly set forth in Example 4 which follows.

The particular chemical composition of the spacer moiety will depend, to some extent, on the nature of the chemical sites available on the respective tridentate members for attachment to the spacer moiety. It will also depend on the availability of organic materials for use as the starting spacer moiety. Typical heteroatoms, including nitrogen, oxygen, sulfur, and phosphorous, may be used in the spacer moiety in addition to carbon atoms. Generally, the spacer moiety will be aliphatic, although aromatic groups may be involved. In the typical divalent chain, where single bonds employing carbon, nitrogen, or oxygen are incorporated into the

spacer moiety, each such atom can be expected to increase the spacer moiety length by about 1.2 to 1.5 Å.

The precise method used to link the tridentate members together, through the spacer moiety, is not critical. What is important is that the tridentate members retain their ability to function effectively in their ultimate intended use following synthesis of the tridentate. For example, a small molecule ligand member must retain the ability to bind to its specific binding partner. This consideration may affect the exact chemical site chosen for connection to the spacer moiety. Ordinarily it is desired to maximize the exposure of the active site or sites of the particular tridentate member which allows for; e.g., a specific binding reaction, to occur.

2. Determination of Spacer Length Requirements

The required spacer length connecting each tridentate member to each other tridentate member must be determined before the tridentate can be synthesized. For example, where a tridentate employing three small molecule ligands is intended for use in a modulated assay, the minimum spacer length between the first and third tridentate members must ordinarily be determined as an initial step. This minimum spacer length establishes the point at which simultaneous binding of the first and third members to their respective specific binding partners can be achieved in the absence of modulation. Below this point, there will be no simultaneous binding.

There are a number of methods known in the art for determining the minimum spacer length required to obtain simultaneous binding of two members of a homobifunctional conjugate to their respective

macromolecular specific binding partners. See, for example, Larsson, P.O., and Mosbach, K., Affinity Precipitation of Enzymes, Elsevier/North Holland Biomedical Press, 98(2), 333-338 (1979), and Green, N.M., Konieczny, L., Toms, E.J., and Valentine, R.C., The Use of Bifunctional Compounds to Determine the Arrangement of Subunits in Avidin, Biochem. J., 125, 781-791 (1971). These same methods can be used to determine the minimum spacer length required between the first and third tridentate members to achieve the same type of simultaneous binding. Specifically, a series of homologs of bifunctional conjugates of various spacer lengths, generally containing only the first and third tridentate members, are synthesized and subsequently analyzed to determine the spacer length at which simultaneous binding is first observed.

The action of simultaneous binding is ordinarily detected by the production of measurable signal which is generated pursuant to the simultaneous binding reaction. One such convenient method for determining minimum spacer length is through the use of standard nephelometric or turbidimetric procedures which detect scattering complexes as a measure of simultaneous binding. These methods require the availability of polyvalent macromolecules to bind to each of the members of the bifunctional conjugate and are useful even where the formation of complexes large enough to form scattering centers is not the ultimate intended use of a particular tridentate. Specifically, bifunctional conjugates which vary only with respect to spacer length (otherwise known as homologs) are brought into contact with the respective multivalent specific binding partners for each member.

A bifunctional conjugate having biotin and theophylline as the two conjugate members may, for

example, be used for a study to detect minimum spacer length. Biotin contains a biologically active alicyclic ring and a short 5-carbon aliphatic tail. In this case, spacer length may be conveniently measured from the biologically active ring structure, with the aliphatic tail being incorporated into the spacer. Homologs of the bifunctional conjugate are prepared and brought into contact with avidin and anti-theophylline antibody. Nephelometric measurements are then taken to detect the amount of complexing, if any. Minimum spacer length is the point at which measurable nephelometric signal is first observed. Optimal spacer length is ordinarily severl bonds longer. The amount of complex formation will ordinarily reach a plateau within several carbon atoms or heteroatoms of the minimum spacer length. It is generally desirable to choose a spacer length near this plateau.

The optimal minimum spacer length does not vary significantly with different proportions of bifunctional conjugate, avidin, and antibody. Moreover, the same minimum spacer length data will generally apply where haptens other than theophylline are used as one of the members of a hapten-biotin bifunctional conjugate, although some slight variation may be observed. Where small molecule ligands other than biotin are incorporated as one of the bifunctional conjugate members, a somewhat greater degree of variation can be expected, due to the greater variation in the size and shape of the different specific binding partners for these ligands as compared to avidin. Consequently, separate homolog studies may be required in some cases to optimize minimum spacer length data. Nevertheless, the study of a theophylline-biotin conjugate, or similar small molecule bifunctional conjugates will provide sufficient optimal minimum spacer length data to at least establish the starting point for

the successful synthesis of any number of tridentate conjugates of the steric hindrance embodiment.

The bifunctional conjugates used to determine minimum spacer length may be synthesized by any one of a number of known prior art methods, as previously indicated. It is preferred that the selected process include the insertion of a compound which is one in a series of homologs in a particular class of compounds. For example, the alkane diamine (NH_2-$(CH_2)_N$-NH_2) class of compounds contains ethane diamine (N=2), propanediamine (N=3), butanediamine (N=4), pentanediamine (N=5), and so forth. Where the synthesis process includes the insertion of one of these homologs, other homologs may be easily substituted to vary the chain length of the spacer connecting the two members of a bifunctional conjugate.

Homologs of two different theophylline-biotin bifunctional conjugates may, for example, be prepared in this way. In the interest of clarity, these two bifunctional conjugates are referred to herein as bifunctional conjugate I and bifunctional conjugate II. Bifunctional conjugate II differs primarily from bifunctional conjugate I in having a carbon atom adjacent to the theophylline moiety rather than the nitrogen atom located adjacent to the theophylline moiety in bifunctional conjugate I.

Initially, primary amine derivatives of theophylline may be prepared from commercially available starting theophylline derivatives as a first step in the synthesis process. Other methods may be used to prepare primary amine derivatives of other haptens. Where bifunctional conjugate I is prepared, an excess of a diamine is refluxed with the starting derivative 8-bromotheophylline. This generally takes place under a

nitrogen atmosphere for a period of two to seventy-two hours, depending upon the particular diamine selected, and results in product I.

Bifunctional conjugate II is prepared from theophylline-8-butyric acid as the starting theophylline derivative and demonstrates the need for activation prior to achieving the desired condensation reaction. Specifically, carbonyldiimidazole (CDI) and N-hyroxysuccinimide (NHS) are used to activate the carboxylic group of the starting derivative theophylline-8-butyric acid before the selected diamine can be inserted. Activation is carried out by dissolving theophylline-8-butyric acid in anhydrous dimethyl-formamide (DMF), followed by heating to about 70°C with the subsequent addition of CDI. The reaction temperature must be maintained at about 70°C, usually for approximately 15 minutes, before it is cooled back to room temperature. The NHS is subsequently added to the cooled reaction mixture and stirred overnight at room temperature. An excess of diamine may then be added to the activated reaction mixture. This results in product II.

The completion of the reaction producing the required primary amine derivative may be determined by a thin layer chromatographic (TLC) analysis of either reaction mixture I or reaction mixture II, using glass TLC plates coated with silica gel and an ultraviolet indicator.

The reaction mixtures must then be evaporated to a small volume under vacuum, with the concentrated reaction mixtures being purified by standard silica gel column chromatography using a gradient chloroform: methanol mixture. The eluted fractions containing the pure primary amine derivatives of theophylline are pooled

and evaporated to dryness in a rotary evaporator. The white-yellowish crystalline solids obtained upon evaporation may be used for the next reaction without further purification. Theophylline monoamine derivatives have a molar absorptivity of about 1.9×10^3 at 295nm in methanol.

Biotin-theophylline conjugate I is prepared by dissolving the crystalline solids containing the primary amine derivatives of theophylline from reaction mixture I in anhydrous DMF and then mixing in the activated N-hyroxysuccinimide ester of caproamidobiotin (biotin-X-NHS), as is schematically shown in Fig. 2. Primary amine derivatives of other haptens may similarly be condensed with biotin-X-NHS or any other chemical moiety containing an activated carboxyl group. This solution is also stirred overnight at room temperature. Biotin-theophylline conjugate II is similarly prepared, with the exception that the crystalline solids containing the primary amine derivatives of theophylline from reaction mixture II are mixed with the N-hydroxysuccinimide ester of biotin (biotin-NHS), as is shown in Fig. 3. In either case, the desired products usually separate out of DMF as white, flocculent solids. The product may be collected on filter paper and purified to a single spot in a TLC test, either by preparative thin layer chromatography or by column chromatography.

Other methods which can similarly be used to determine minimum spacer length include enzyme channeling and energy transfer schemes. The same series of homologs of bifunctional conjugates used in the NIIA analysis, may be brought into contact with the respective labeled specific binding partners for each of the first and third members. These studies are similar to typical modulated assay systems, except that there is no free analyte and

no modulator. Signal, generated by the proximity labels, will again begin to be observed at the minimum spacer length which allows for simultaneous binding of the labeled macromolecules, with optimal spacer length ordinarily being a little longer than minimum spacer length.

Where either the first or third tridentate member does not have a specific binding partner, spacer length between the first and third tridentate members is not as critical, because there is no requirement of simultaneous binding between these two members. For example, where one of these members is a second proximity label, a specific binding partner conjugated to a first proximity label can easily bind to its corresponding tridentate member due to the small molecular size of the second proximity label (tridentate member). Where one of the first or third tridentate members is a macromolecule to which has been conjugated a plurality of second proximity labels, there will ordinarily be some minimum spacer length requirement to enable the labeled specific binding partner to bind to its corresponding small molecule ligand member, but the minimum spacer length will be shorter than that required for simultaneous binding of two specific binding partners. In this instance, similar homolog studies can be performed wherein one member of the bifunctional conjugate homolog is the intended macromolecular tridentate member.

It should be noted that the section of the spacer moiety connecting two tridentate members is ordinarily not as straight or rigid as the spacer moiety connecting two bifunctional conjugate members. This is due, in part, to the tetrahedral spatial arrangement of the four bonds of the carbon atom ordinarily located at the hinge position of the spacer. See Fig. 7. For this reason, an increase in spacer length on the order of

about 10% to 20% should ordinarily be added to the minimum optimal spacer length observed where a bifunctional conjugate employing the intended first and third tridentate members is used to approximate the simultaneous binding action of the same members in the tridentate conjugate.

The same data generated to establish minimum spacer length for simultaneous binding between two small molecule ligand members of a universal tridentate can also be used to determine the relative positioning of the second tridentate member of a tridentate intended for use in a competitively modulated immunoassay. The section of spacer moiety connecting the second member with the remaining tridentate member(s) of which modulation is desired must be less than the minimum spacer length which allows simultaneous binding. Where the tridentate members are all small molecule ligands, the distance between the first and third tridentate members will be longer than the distance from the second (modulating) member to the first and/or third members, depending on which of the remaining members is desired to be modulated.

In the targeted labeling embodiment, the minimum spacer length requirement is between the intended label and the targeted macromolecule. Where this member is a small molecule ligand, the length of the section of the spacer moiety connecting the intended label to the reactive member must be of a sufficient length to enable the attached intended label to bind to its specific binding partner without incurring steric hindrance from the targeted macromolecule. Generally, the same data establishing the minimum spacer length for simultaneous binding between two small molecule ligands or between a small molecule ligand and a macromolecule may also be

used here, although some slight experimentation with homologs may be required to fully optimize results. Where the third tridentate member is also a macromolecule, like the targeted macromolecule, spacer length is not generally critical. This is particularly true, because the present invention enables targeted conjugation such that the active site of e.g., an enzyme, can be specifically located away from the site of conjugation to; e.g., a solid support.

3. Building the Tridentate Conjugate

The three tridentate members may generally be conjugated together using traditional organic synthesis techniques known in the art. The tridentate of the present invention, however, requires the incorporation of a carefully selected spacer moiety in order to control the desired functional attributes of the tridentate. Consequently, it has been found that it is preferable to begin synthesis of the tridentate with a starting spacer moiety around which the remainder of the tridentate is built. Various sections of the starting spacer moiety may be lengthened, as desired, during the synthesis process.

It is preferred that the starting spacer moiety be an organic molecule having three chemical functional groups which can be suitably and individually derivatized. More preferably, the three chemical groups will all be different functional groups. Where two or more of the chemical groups are the same functional group, one or more of these same chemical groups must be capable of: (1) being protected while the other identical group(s) is(are) being derivatized; and, (2) subsequ ntly being deprotected without causing chemical modification to the

remainder of the partially synthesized tridentate or to the protected functional group itself.

Typical functional groups which may be suitably derivatized include amino groups ($-NH_2$), carboxyl groups (-COOH) and mercapto groups (-SH). Amino groups and carboxyl groups react with each other to unite two molecules in a typical condensation reaction. Normally, the carboxyl group must be activated prior to the condensation reaction. Mercapto groups react with other mercapto groups as well as maleidoimidyl groups to ultimately link two molecules together via covalent bonding.

The starting spacer moiety may be a synthetic molecule, or it may be a molecule found in nature. Naturally occurring amino acids generally provide good starting spacer moieties for synthesis of the tridentate. Almost all of the naturally occurring amino acids are α-amino acids which, by definition, contain both an amino group and a carboxyl function at the α-carbon position of the amino acid. Some of these amino acids also have an additional functional group at the ω-position, or terminal carbon, of the amino acid. Lysine, for example has a second amino group at the ω-position. By contrast, there is a second carboxyl function at the ω-position of glutamic acid.

Mercapto amino acids, such as cysteine, are particularly suitable for use as starting spacer moieties, because they possess three different chemical functional groups, namely, amino, carboxyl, and mercapto groups. Lysine is also a preferred amino acid for use as a starting spacer group, due to its availability and relative inexpense. Other preferred amino acids include

glutamic acid, as well as other naturally occurring amino acids such as tyrosine and serine.

As noted, both lysine and glutamic acid contain two identical functional groups. In order to individually derivatize the chemical groups on these and other starting spacer moieties having two identical functional groups, one of these functional groups must be protected while the other is being derivatized. Suitable protecting groups for carboxyl functions include, for example, benzyl esters and tertiary-butyl ester groups. Where the protection of an amino group is desired, carbobenzoxy esters, benzoyloxycarbonylphthalyl, or 9-fluorenyl-methyloxycarbonyl may, for example, be used. Other protecting groups for these and other functions are known in the basic synthesis art. The protecting group must be capable of removal (i.e., deprotection) without harm to the functional group or to the rest of the tridentate.

The order in which the intended tridentate members are attached to the starting spacer moiety is ordinarily not critical. In these instances, the order of attachment will generally be mandated by considerations of convenience. In certain instances, however, such as where one of the tridentate members is a solid support conjugated to a plurality of proximity labels, the solid support member should be attached last. For example, in synthesizing the tridentate(s) shown in Figure 11, partially synthesized tridentates (having the theophylline and DNP moieties already attached) may be conjugated to a solid support at the same time as the isoluminol proximity labels. The ratio of tridentate/proximity label can generally be controlled by the respective amounts of these compounds added to the reaction medium.

A. Cyclic Acid Anhydrides as Starting Spacer Moieties

Cyclic acid anhydrides provide particularly good starting spacer moieties, due to their ability to "self-protect" one of the two carboxyl functions generally used for attaching tridentate members to these starting spacer moieties. For example, the two carboxyl functional groups of glutamic acid appear as the anhydride function of pyroglutamic acid.

The primary amino group of an intended first member can react with the anhydride function of the pyro-glutamic acid to yield a conjugated glutamic acid, as is shown in Fig. 4. Only one of the carboxyl groups of the anhydride function will add to the primary amino group, thus liberating the remaining carboxyl group in the form of a free carboxyl function. The free carboxyl group can later be separately derivatized through a condensation reaction with the primary amino group of another intended member. The condensation reaction can take place immediately after attachment of the first member, or, for example, following attachment of the second tridentate member to the amino function of glutamic acid, as is shown in Fig. 4. The amino function of pyroglutamic acid is preferably conjugated to a protecting group, such as a carbobenzoxy (CBZ) group, in order to prevent the amino group of pyroglutamic acid from polymerizing with the liberated carboxyl function.

Ordinarily, it is difficult to control which one of the two carboxyl groups of an anhydride function will react with a primary amine. This generally results in the formation of two different isomers, such as those shown following addition of the first tridentate member in Fig. 4. The section of the spacer moiety connecting the first member to the remaining tridentate members is

one bond longer in one isomer than it is in the other. The opposite holds true with respect to the tridentate member which is attached to the liberated carboxyl function, in this case, the third tridentate member. For example, in the completed tridentate, shown in Fig. 4, the section of the spacer moiety connecting the first member to the second member is seven bonds in one isomer and six bonds in the other isomer. The opposite holds true with respect to the section of spacer moiety connecting the third member to the second member; i.e., it is six bonds long in one isomer and seven bonds long in the other. The section of the spacer moiety connecting the first and third members (the members attached through the carboxyl functions) is the same in both isomers.

Another useful cyclic anhydride is S-acetyl-mercaptosuccinic anhydride. The primary amino group can react with the anhydride function of S-acetyl-mercaptosuccinic anhydride to yield a substituted, S-protected succinic acid, wherein the primary amino group of the intended first member adds to one of the carboxyl groups of the anhydride. See Fig. 5. This frees the remaining carboxyl group, in the form of a free carboxyl function to which one of the remaining tridentate members can be relatively easily attached. Following deprotection, the mercapto group can be attached to yet another tridentate member, as is shown in Fig. 5.

As with pyroglutamic acid, and other acid anhydrides, two isomeric tridentates are ultimately formed. As a result, the section of spacer moiety connecting each of the two members attached through the carboxyl groups varies by one bond. Ordinarily, this slight variation in spacer length does not affect the performance of the tridentate. In the unusual

circumstance where it is desired to use only one particular isomer, the desired isomer can be separated out of the mixture at an early stage, following the initial addition reaction, using standard separation procedures.

The various section lengths of the spacer moiety can be easily controlled or varied using methods similar to those earlier set forth for varying the spacer length connecting two bifunctional conjugate members. For example, a series of diamine homologs is particularly useful in adding spacer length to an intended tridentate member where a primary amine is necessary for attachment to the starting spacer moiety. Adjustments in spacer section length are ordinarily made prior to attachment to the starting spacer moiety.

B. Carbobenzoxylysine As Starting Spacer Moiety

A good example of a starting spacer moiety requiring protection of one of two identical functional groups is lysine. The commercially available ω-carbobenzoxylysine (ω-CBZ-lysine) provides a good "preprotected" starting spacer moiety for the synthesis of a variety of tridentates. The carbobenzoxy protecting group is attached to the second amino function at the ω-position of the lysine molecule and is removed only after the α-amino function has been derivatized. It is often convenient, but not necessary, to attach the intended first member to the α-amino group of ω-CBZ-lysine.

The preprotected ω-CBZ-lysine may be used as the starting spacer moiety in the synthesis of tridentates employing the steric hindrance embodiment of the present invention as well as in the synthesis of tridentates employing the targeted labeling embodiment. For example,

a universal biotin-theophylline-DNP tridentate, useful in competitively modulated assays for theophylline such as the previously discussed NIIA shown in Fig. 1, may be conveniently synthesized from ω-CBZ-lysine. The synthesis of this tridentate is schematically shown in Fig. 6.

The first tridentate member, biotin, may be attached to the α-amino group of ω-CBZ-lysine (spacer) by utilizing the commercially available activated form of biotin, biotin-NHS. (Biotin-X-NHS may, for example, be used where a longer spacer is desired). The ω-CBZ-lysine is first dissolved in a bicarbonate solution and then heated to boiling to effect dissolution. Upon being cooled back to room temperature and then filtered, biotin-NHS (activated first member) is added to the solution, whereupon the activated carboxyl group of biotin readily condenses with the α-amino group of lysine, as is shown in Fig. 6. Solids of the derivatized biotinyl-CBZ-lysine (first member-spacer) may then be collected by standard filtration methods. It is ordinarily unnecessary to further purify the biotinyl-CBZ-lysine before proceeding with attachment of the second member.

The second tridentate member, theophylline is attached, in the form of a primary amine, to the activated α-carboxyl group of the derivatized lysine. The attachment of theophylline may take place either before or after conjugation of the protected ω-amino group. The desired primary amine derivative of theophylline may be prepared from 8-bromotheophylline as previously set forth as the first step in the synthesis of bifunctional conjugate I. The sections of the spacer moiety connecting theophylline to the other two tridentate members may be controlled through the selection of the particular diamine used to pr pare the

primary amine derivative of theophylline. Ethylenediamine may, for example, be used to prepare theophylline-ethylenediamine. The desired primary amine derivative of theophylline, thus prepared, is dissolved in anhydrous DMF in preparation for its conjugation to the starting spacer moiety.

The biotinyl-CBZ-lysine (first member-spacer) solids, obtained from the derivatization of the α-amino group of the lysine spacer, are dissolved in anhydrous DMF and heated to approximately 70°C whereupon the α-carboxyl group of the lysine spacer is activated by the addition of CDI. After cooling back to room temperature, the predissolved selected primary amine derivative of theophylline (second member), e.g., theophylline-ethylenediamine, is added to the solution. The activated α-carboxyl group of the lysine spacer readily condenses with the primary amine derivative of theophylline, as is shown in Fig. 6, forming a precipitate containing the derivatized biotin-theophylline-CBZ-lysine (first member-second member-spacer) which may then be collected by standard filtration methods and dried. The biotin-theophylline-CBZ-lysine conjugate may be purified by subjecting the precipitate to separation chromatography on a silica gel column using a gradient chloroform:methanol mixture.

The carbobenzoxy protecting group must be removed from the ω-amino group of the twice derivatized lysine spacer before the ω-amino group can be conjugated to the third member. Removal of the carbobenzoxy protecting group can be effected in a number of ways. One convenient way is to dissolve the isolated biotin-theophylline-CBZ-lysine (first member-second member-spacer) in a commercially available 30% (wt.%, density 1.262) mixture of hydrobromic acid in acetic acid. The

acid mixture is then diluted with deionized water and subsequently neutralized with solid sodium bicarbonate. The ω-amino group of the now deprotected biotin-theophylline-lysine conjugate (first member-second-member spacer) may be derivatized using an activated carboxyl group at the terminal end of the intended third member.

A terminal carboxyl group may be attached to the intended third member, DNP, by reacting Bis-aminocaproic acid with 2,4-dinitro-fluorobenzene (a DNP precursor also known as Sanger's reagent) to form DNP-Bis-aminocaproic acid. This reaction reaches completion at room temperature in about two hours. As is seen in Fig. 6, Bis-aminocaproic acid contributes 14 atoms to the common section of spacer moiety connecting DNP to the other tridentate members. The length of this section of the spacer can easily be controlled using an alternate ω-amino acid other than Bis-aminocaproic acid. For example, 5-aminopentanoic acid can be condensed with 6-aminocaproic acid to form a 13 atom spacer insert.

The DNP-Bis-aminocaproic acid (third member-spacer insert) may be isolated and purified by evaporating the reaction mixture to dryness, redissolving the residue in deionized water, acidifying the solution with hydrochloric acid, and then extracting the DNP-Bis-aminocaproic acid (third member-spacer insert) with ethyl acetate. The ethyl acetate may then be eliminated and the DNP-Bis-aminocaproic acid further purified using standard silica gel column chromatographic procedures.

CDI and NHS are used to activate the terminal carboxyl group of the DNP-Bis-aminocaproic acid (third member-spacer insert) by forming the reactive NHS ester of the acid. This reaction tak s place relatively quickly in anhydrous chloroform. The chloroform solution is

evaporated to near dryness and then brought back up to volume with anhydrous DMF, whereupon it is added to the previously set aside solution containing the deprotected biotin-theophylline-lysine (first member-second member-spacer). The condensation reaction between the exposed ω-amino group of the lysine spacer moiety and the activated carboxyl group of the DNP-Bis-aminocaproic acid (activated third member-spacer insert) occurs quite readily thus attaching the third member of the biotin-theophylline-DNP tridentate (first member-second member-third member).

The same commercially available preprotected ω-CBZ-lysine may also be used as the starting spacer moiety for a tridentate employing the targeted labeling embodiment of the present invention. The synthesis of a phenylboronic acid-nitrophenyl azido-biotin conjugate, for example, is shown in Fig. 8.

As with the synthesis of the biotin-theophylline-DNP tridentate, the carbobenzoxy protecting group is removed only after the α-amino function of the CBZ-lysine has been derivatized. The reactive azide member is first attached to the CBZ-lysine by reacting 3-nitro-4-fluorolphenylazide with the α-amino group of CBZ-lysine as is shown in Fig. 8. The azide moiety readily attaches to the CBZ-lysine spacer, producing azide-CBZ-lysine (reactive member-spacer).

The α-carboxyl function of the derivatized CBZ-lysine is generally activated with CDI and NHS prior to further derivatization at the α-carboxyl position. A boronic acid moiety (guiding member) having a primary amine available for conjugation may then be attached to the activated α-carboxyl function of the derivatized

azide-CBZ-lysine, through a standard condensation reaction. See Fig. 8.

The third tridentate member is attached to the twice-derivatized boronic acid-azide-CBZ-lysine (guiding member-reactive member-spacer) only after the carbobenzoxy protecting group is removed. This can again be accomplished by dissolving the boronic acid-azide-CBZ-lysine (guiding member-reactive member-spacer) in a commercially available 30% (wt.%, density 1.262) mixture of hydrobromic acid in acetic acid. This acid mixture may be diluted with deionized water and subsequently neutralized with sodium bicarbonate. The ω-amino group of the now deprotected boronic acid-azide-lysine conjugate (guiding member-reactive member-spacer) may be derivatized using an activated carboxyl group at the terminal end of the intended third member.

Where biotin is the intended third member, the commercially available biotin-NHS (5 atoms added to spacer) or biotin-X-NHS (12 atoms added to spacer) may be used. Alternatively, Bis-caproamidobiotin (biotin-X-X-NHS) may be conveniently used where 19 atoms are desired to be added to the spacer. All of these "preactivated" biotin derivatives readily condense with the ω-amino group of the lysine starting spacer moiety to yield the desired boronic acid-azide-biotin (guiding member-reactive member-intended label) tridentate conjugate. The tridentate conjugate shown in Fig. 9 results where biotin-X-X-NHS is employed in the final derivatization step.

C. Other Tridentates

Still other tridentates of the present invention can be synthesized using CBZ-lysine, cyclic acid

anhydrides, or other suitable starting spacer moieties. For example, the tridentates shown in Fig. 10 may be readily synthesized for use in energy transfer assays employing the steric hindrance embodiment of the present invention wherein one of the first or third tridentate members is a proximity label. The different energy donor proximity labels may conveniently be attached as tridentate members. It will be apparent to those skilled in the art how to make these and other tridentate conjugates in light of the foregoing discussion.

Example 1

Synthesis of Bifunctional Conjugate I

The synthesis of bifunctional conjugate I using hexanediamine (N=6) as the variable for insertion into the spacer is schematically shown in Figure 2. The N=6 homolog of bifunctional conjugate I was produced in the following manner:

An excess of hexanediamine (NH_2-$(CH_2)_6$-NH_2) was refluxed with 8-bromotheophylline under a nitrogen atmosphere for a period of 24 hours. The end of the reflux reaction was determined by TLC analysis of the reaction mixture, using glass TLC plates coated with silica gel, and using an ultra-violet indicator.

The reaction mixture was then evaporated to a small volume under vacuum. The concentrated reaction mixture was mixed with a small quantity of silica gel and dried on a hot plate with the silica gel-sample mixture then being carefully loaded onto the top of a silica gel column using chloroform as the starting eluant. The column was eluted with solvent containing varying amounts of methanol in chloroform. When the gradient composition

reached 20% methanol in chloroform, the column was eluted with a mixture containing 20% methanol, 4% ammonia, and 76% chloroform. The fractions containing the pure N-(8'-theophylline)-6-aminohexylamine were pooled and evaporated to dryness in a rotary evaporator. White-yellowish crystalline solids were used for the next reaction without further purification.

Equimolar quantities of the N-(8'-theophylline)-6-aminohexylamine crystalline solids were dissolved in anhydrous DMF, then mixed with the corresponding molar quantity of biotin-X-NHS and stirred overnight at room temperature. The desired products separated out of DMF as white, flocculent solids, and were collected on filter paper and purified to a single spot in a TLC test by column chromatography.

The chain length of the spacer moiety is controlled by the diamine (NH_2-$(CH_2)_N$-NH_2) selected for use in the synthesis of the bidentate. For example, where hexanediamine is selected for the first synthesis step, six carbon atoms are contributed to the spacer moiety chain length, as shown in Fig. 2. In this instance, the approximate length of the spacer moiety is 26.0 Å. The chain length obtained from the insertion of various diamines into the spacer of bifunctional conjugate I, using a procedure similar to that used to prepare the N=6 homolog, is shown in Table I.

TABLE I

Length of Spacer Moiety in Bifunctional Conjugate I Homologs

Diamine	Total Number of Atoms in Spacer	Approximate Spacer Length (Å)
N=2	16	21.0
N=3	17	22.2
N=4	18	23.5
N=5	19	24.8
N=6	20	26.0
N=7	21	27.3
N=8	22	28.5

Example 2

Determination of Minimum Spacer Length

Minimum spacer length was determined by detecting the amount (rate) of signal generated by each of the conjugates identified in Table 1 in the presence of anti-theophylline antibody and avidin. The purpose of these measurements was simply to determine minimum spacer length for optimal binding at stoichiometric proportions of bifunctional conjugate, antibody, and avidin.

The reagents were prepared as follows: monoclonal antibody against theophylline was diluted 1:13.3 in ICS™ Diluent (Beckman Instruments, ICS™ Reagent). Avidin, purchased from Boehringer Mannheim, was dissolved in ICS diluent at a concentration of 0.13 mg/mL. Various dilutions

of each of the bidentate conjugates listed in Table 1 were dissolved in 0.1M phosphate buffer, pH 5.5.

Nephelometric measurements were taken on an ICS™ nephelometer (Beckman Instruments) by placing 600μL of ICS Buffer (Beckman Instruments, ICS™ Reagent) into an ICS™ vial (Beckman Instruments), and injecting 42μL of antibody solution and 42μL of avidin solution. After the injection transient subsided and the base-line was obtained, 42μL of the bidentate conjugate were added and the instrument triggered to record the peak rate signal.

The results for N=2 through N=8 are reported in Figure 12. In Figure 12, the units on the horizontal axis represent bidentate concentration based on absorbance at 295nm. The units on the vertical axis are ICS™ rate units, obtained using the ICS™ Manual Mode Card M33 (Beckman Instruments). For high rate signals, above 2000 units, the ICS™ lower gain card was used and the results calculated for M33 gain.

As is seen in Figure 12, the lowest homolog of bifunctional conjugate I (N=2), having 17 bonds (16 atoms) between the theophylline ring carbon and the alicyclic ring carbon of biotin failed to yield measurable complex formation. The next higher homolog (bifunctional conjugate I, N=3) began to show measurable complexing. The higher homologs (N=4 through N=8) produced correspondingly higher signal until a plateau was reached at N=8. This study shows that a minimum spacer length of approximately 22.2 Å is required in order to produce signal where theophylline and biotin are employed as the first and third tridentate members. Optimally, the spacer length should be at least about 23.5 Å (bifunctional conjugate I, N=4), and more preferably about 26.0 to about 28.5 Å (bifunctional conjugate I, N=6 to N=8, or 20 to 22 atoms in the spacer).

Example 3

Synthesis of Bifunctional Conjugate II

A second bifunctional conjugate was prepared to confirm the results obtained for bifunctional conjugate I and to demonstrate an alternate synthesis method. The synthesis of bifunctional conjugate II using hexanediamine as the variable for insertion into the spacer is schematically shown in Figure 5. The N=6 homolog of bifunctional conjugate II was produced in the following manner:

Theophylline-8-butyric acid was dissolved in anhydrous DMF, then heated to about 70°C with the subsequent addition of an equimolar quantity of CDI. The reaction temperature was maintained at about 70°C for approximately 15 minutes, then allowed to cool to room temperature. An equimolar quantity of NHS was then added to the cooled reaction mixture and stirred overnight at room temperature. An approximate three to six molar excess of hexanediamine (H_2N-$(CH_2)_6$-NH_2) was then added to the reaction mixture. The completion of reaction was determined by TLC analysis of the reaction mixture, using TLC plates coated with silica gel and an ultraviolet indicator.

The reaction mixture was then evaporated to a small volume under vacuum. The concentrated reaction mixture was mixed with a small quantity of silica gel and dried on a hot plate with the silica gel-sample mixture then being carefully loaded onto the top of a silica gel column using chloroform as the starting eluant. The column was eluted with solvent containing varying amounts of methanol in chloroform. When the gradient composition reached 20% methanol in chloroform, the column was eluted with a mixture containing 20% methanol, 4% ammonia, and 76% chloroform. The fractions containing the pure 6-(8'-theophylline butyric

carboxamido)-hexylamine were pooled and evaporated to dryness in a rotary evaporator. White-yellowish crystalline solids were used for the next reaction without further purification.

An equimolar quantity of the 6-(8'-theophylline butyric carboxamido)-hexylamine was dissolved in anhydrous DMF, then mixed with the corresponding molar quantity of biotin-NHS and left stirred overnight at room temperature. The desired products separated out of DMF as white, flocculent solids, and were collected on a filter paper and purified to a single spot in a TLC test by preparative thin layer chromatography.

The N=5 homolog of bifunctional conjugate II was prepared using an identical procedure with the exception that pentanediamine was used in place of hexanediamine. The spacer lengths for the two homologs are set forth in Table II.

TABLE II

Length of Spacer Moiety in Bidentate Conjugate II Homologs

Diamine	Total Number of Atoms in Space	Approximate Spacer Length (Å)
N=5	16	21.0
N=6	18	22.2

The N=2 homolog of bifunctional conjugate I is equivalent to the N=5 homolog of bifunctional conjugate II (21.0 Å). The N=3 and N=6 homologs of bifunctional conjugates I and II, respectively, are also equivalent (22.2 Å). The N=5 homolog of the second series, like the N=2 homolog of the first series failed to yield measurable complexing. Comparable signals were obtained,

however for the N=6 homolog of bifunctional conjugate II and the N=3 homolog of bifunctional conjugate I. This confirms that at least a 22.2 Å spacer moiety is required to achieve simultaneous binding of the first and third tridentate members to their specific binding partners where theophylline and biotin or similar haptens and/or small molecules are chosen as the first and third tridentate members.

Example 4

Synthesis of biotin-theophylline-DNP Tridentate

A universal biotin-theophylline-DNP tridentate conjugate, for use in competitively modulated immunoassays for theophylline, was synthesized using CBZ-lysine as the starting spacer moiety. The optimal minimum spacer length data from the biotin-theophylline bifunctional conjugate I homolog study was used as the starting point for designing the biotin-theophylline-DNP tridentate. Specifically, an optimal minimum spacer length of about 26.0 to about 28.5 Å, or 20 to 22 atoms in the spacer, was established for simultaneous binding of the biotin and theophylline members of a bifunctional conjugate. (See Example 2.) A 10 to 20% increase, or about 22 to 26 atoms, was thus believed to be optimal for obtaining simultaneous binding of the biotin and DNP (first and third) members of the tridentate in the absence of modulation. Biotin was selected to be the modulated member, with a much shorter spacer length, on the order of 12 atoms, being chosen for the section of the spacer moiety connecting theophylline (modulating member) with biotin.

Attachment of First Tridentate Member

The starting spacer group, ω-carbobenzoxy-lysine (CBZ-lysine), was added to a 10% sodium bicarbonate solution which was heated to boiling to effect dissolution and then cooled back to room temperature. The cooled solution was then filtered through fluted filter paper. An equimolar quantity of biotin-NHS, containing the intended first member biotin, was added to the solution and stirred at room temperature for about 24 hours. White solids of biotinyl-CBZ-lysine formed during the course of the reaction and were collected by standard filtration methods. This crude preparation of biotinyl-CBZ-lysine was used in the further preparation of the tridentate without being subjected to additional purification steps.

Attachment of Second Tridentate Member

The biotinyl-CBZ-lysine was dissolved in anhydrous DMF and the mixture heated to approximately 70°C whereupon the carboxyl group of the lysine moiety was activated by the addition of CDI. The activation process was allowed to proceed for about 15 minutes before the solution was cooled back to room temperature. The cooled mixture was then stirred at room temperature for an additional 30 minutes. An equimolar quantity of theophylline-ethylenediamine was first dissolved in DMF and then added to the cooled mixture, which was left stirring overnight at room temperature. A white precipitate containing biotin-theophylline-CBZ-lysine formed overnight and was collected by standard filtration methods and dried.

The precipitate contained a relatively minor unidentified contaminant which was separated out on a

silica gel column using a gradient chloroform:methanol mixture. The precipitate was mixed with a small quantity silica gel and then carefully loaded onto the top of a silica gel column using chloroform as the starting eluant. The column was eluted with solvent containing varying amounts of methanol in chloroform, until the gradient composition reached 20% methanol in chloroform. The first compound to be eluted from the column was the biotin-theophylline-CBZ-lysine which was collected and evaporated to dryness, yielding a white crystalline powder. Ultraviolet absorption and TLC analysis of the white crystalline powder confirmed that the product contained both the biotin and theophylline moieties.

Attachment of Third Tridentate Member

In order to attach the third tridentate member, three steps were required: (1) the ω-amino group at the third member position of the lysine moiety had to be deprotected; i.e., by the removal of the CBZ group; (2) a carboxyl group had to be attached to the end of the intended third member; and, (3) the attached carboxyl group had to be activated in order to react with the free amino group at the third member position of the lysine moiety.

The carbobenzoxy protecting group was removed from the ω-amino group of the lysine spacer by dissolving the white crystalline powder in an excess of commercially available 30% (wt.%, density 1.262) hydrobromic acid in acetic acid. The acid mixture was diluted to approximately 50 times its original volume with deionized water and then neutralized with solid sodium bicarbonate until the pH of the solution was brought up to about 8-9. The neutralized solution was then set aside while the intended third member was prepared for attachment.

A terminal carboxyl group was attached to the intended third member (DNP) of the tridentate by reacting Bis-aminocaproic acid with 2,4-dinitrofluorobenzene (Sanger's reagent). Specifically, an excess of 2,4-dinitrofluorobenzene was added to Bis-aminocaproic acid which had previously been dissolved in a 1M. solution of sodium bicarbonate, and allowed to react at room temperature for about two hours. The reaction mixture was evaporated to dryness in a rotary evaporator under reduced pressure. The residue was then redissolved in deionized water and acidified to about pH 1 with 28% (wt.%) hydrochloric acid. Yellow precipitate containing DNP-Bis-aminocaproic acid formed and was extracted with ethyl acetate, which was then eliminated by using a rotary evaporator, leaving a yellow solid. The solid was further purified using a standard silica gel column chromatographic procedure similar to that outlined above for the purification of biotin-theophylline-CBZ-lysine.

Dicyclohexylcarbodiimide (DCCI) and NHS were used to activate the terminal carboxyl group of the DNP-Bis-aminocaproic acid by forming the reactive NHS ester of the acid. The purified DNP-Bis-aminocaproic acid was first dissolved in anhydrous chloroform, to which DCCI and NHS were subsequently added. The NHS ester of DNP-Bis-aminocaproic acid quickly formed within about 60 minutes. TLC analysis indicated the ester to be pure, but the ester proved difficult to crystallize, nonetheless. Consequently, the solution was evaporated to near dryness and then brought back up to volume with anhydrous DMF.

The DMF solution containing the activated NHS ester of DNP-Bis-aminocaproic acid was then added to the previously set aside solution containing the deprotected theophylline-biotin-lysine. The condensation reaction

between the exposed ω-amino group of the lysine spacer moiety and the activated carboxyl group of the DNP-Bis-aminocaproic acid occurred quite readily at room temperature, thus attaching the third member to the tridentate. The structure of the completed tridentate is shown in Fig. 7.

Example 5

Bidentate Conjugate I in NIIA for Theophylline-amine

A NIIA type of assay for theophylline-amine was successfully run using the biotin-theophylline-DNP tridentate from Example 4. Free theophylline-amine, from standardized solutions, competed with the second tridentate member for a limited quantity of anti-theophylline antibody. Increasing concentrations of theophylline-amine resulted in decreased modulation and increased nephelometric signal. The same tridentate and assay conditions can be used to test for theoplylline.

The reagents were prepared as follows: Monoclonal antibody against theophylline was diluted 1:20 in ICS™ Diluent (Beckman Instruments, ICS™ Reagent). Rabbit anti-DNP antiserum, purchased from Miles Laboratories, was dialyzed in ICS Diluent prior to use. Avidin, purchased from Boehringer Mannheim, was dissolved in ICS diluent at a concentration of 0.25 mg/mL. The tridentate conjugate was dissolved in ICS Diluent at a concentration of 2.06×10^{-8} moles/mL. Theophylline-amine was diluted in ICS diluent to a concentration of 2.8×10^{-8} moles/mL. Similar solutions were made up to final concentrations of 1.4×10^{-8} and 0.56×10^{-8} moles/mL, respectively.

A 138μL aliquot of monoclonal anti-theophylline antibody solution was deposited in a test tube, followed by the addition of 18.4μL of theophylline-amine at 2.8 x 10^{-8} moles/mL. The resulting mixture was then stirred for two minutes at room temperature. A 30μL aliquot of the tridentate conjugate solution was then added to the test tube, which was mixed and then left standing for about two minutes.

Nephelometric measurements were taken on an ICS™ nephelometer (Beckman Instruments) by placing 600μL of ICS Buffer (Beckman Instruments, ICS™ Reagent) into an ICS™ vial (Beckman Instruments), and injecting 31μL of the above mixture, and 50μL of anti-DNP antiserum solution. An instrument gain setting of Manual Mode M33 was used. After the injection transient subsided and the baseline was obtained, 10μL of the avidin solution was added and the instrument triggered to record the peak rate signal.

The same procedure was then repeated using I8.4μL of the theophylline-amine solutions at 1.4 x 10^{-8} and 0.56 x 10^{-8} moles/L and 18.4μL of ICS Diluent (zero dose test). The results are set forth in Table III.

TABLE III

Theophylline-amine Concentration (moles/mL)	Rate Units
0	158
0	171
0	165
0.56×10^{-8}	240
0.56×10^{-8}	265
0.56×10^{-8}	218
1.40×10^{-8}	365
1.40×10^{-8}	389
1.40×10^{-8}	361
2.80×10^{-8}	648
2.80×10^{-8}	638
2.80×10^{-8}	610

Example 6

Enzyme Channeling Using Universal Tridentate

The same biotin-theophylline-DNP tridentate can be used to perform a competitively modulated enzyme channeling assay. For example, a first enzyme, such as hexokinase, is attached to either avidin or anti-DNP antibody. A second enzyme, such as G6PDH, is attached to the other specific binding partner. Free theophylline or theoplylline-amine, contributed by a test sample or a calibration standard, modulates enzyme channeling by diverting anti-theophylline antibody away from the second (modulating) member of the tridentate.

Preparation of HK-Avidin Conjugate

Hexokinase is thiolated by suspending the HK enzyme in 0.1M phosphate buffer, pH 7.5, containing 20%

(v/v) DMF, and incubating the suspension with S-acetyl-mercaptosuccinic anhydride. After allowing the reaction to reach completion, the thiol groups of the hexokinase are deblocked by treating the mixture with 1.0M hydroxylamine, pH 7.5. The thiolated hexokinase can be isolated by either passing the mixture through a Sephadex® G-50 (bead-formed, cross-linked dextran, Pharmacia, Uppsala, Sweden) column or by standard dialysis methods.

Avidin is also suspended in a 0.1M phosphate buffer solution, pH 7.5, containing 20% (v/v) DMF, and then treated with meta-maleiimidobenzoyl-N-hydroxy-succinimide (MBS-NHS). The reaction mixture can then be passed through a Sephadex® G-50 column to isolate the MBS-labeled avidin.

Equimolar quantities of the thiolated hexokinase and MBS-labeled avidin are then incubated to obtain the HK-avidin conjugate.

Preparation of G6PDH-Anti-DNP Antibody

The G6PDH-labeled anti-DNP antibody is prepared in much the same manner as the avidin-HK conjugate. The enzyme is first thiolated by suspending the G6PDH in 0.1M phosphate buffer, pH 7.5, containing 20% (v/v) DMF, and incubating the suspension with S-acetyl-mercaptosuccinic anhydride. After allowing the reaction to reach completion, the thiol groups of the G6PDH are deblocked by treating the mixture with 1.0M hydroxylamine, pH 7.5. The thiolated G6PDH can be isolated by either passing the mixture through a Sephadex® G-50 column or by standard dialysis methods.

Anti-DNP antibody is also suspended in a 0.1M phosphate buffer solution, pH 7.5, containing 20% (v/v)

DMF, and then treated with MBS-NHS. The reaction mixture can then be passed through a Sephadex® G-50 column to isolate the MBS-labeled anti-DNP antibody.

Equimolar quantities of the thiolated G6PDH and MBS-labeled anti-DNP antibody are then incubated to obtain the G6PDH-anti-DNP antibody conjugate.

Determination of Optimum Reagent Concentrations for Enzyme Channeling

It is ordinarily desirable to determine the optimum reagent concentrations for the production of signal. This is the point at which: (1) maximum signal is obtained, in the absence of modulation; with, (2) minimum use of expensive reagents.

The following reagents are used:

Incubation buffer: 50 mM Bicin, pH 8.4, 100mM KCl, 0.2% bovine serum albumin (BSA), 0.05% sodium azide.

Tridentate solution: tridentate conjugate dissolved in incubation buffer to a concentration equivalent to about 1µg/mL theophylline.

Proximity label solution: equimolar quantities of HK-avidin and G6PDH-anti-DNP antibody suspended in incubation buffer. Various dilutions are prepared.

Antibody solution: anti-theophylline antibody suspended in incubation buffer. Various dilutions are prepared.

Substrate mixture: 50 mM Bicin, pH 8.4, 100mM

KCl, 6mM $MgCl_2$, 3mM ATP, 3mM NAD+, 40mM glucose, and 40% glycerol.

Initially, 100µL of the tridentate solution and 100µL of the proximity label solution are introduced into 800µL of the substrate mixture. The rate of NADH production is a measure of the amount of glucose-6-phosphate, generated by the HK, which has been acted upon by the G6PDH before escaping into bulk solution; i.e., the rate of enzyme channeling. This reaction can be measured using a suitable fluorometer with 450nm detection wavelength and 340nm excitation wavelength settings. The measurements are repeated using increasing dilutions of the proximity label solution until the rate of NADH production begins to decrease. This establishes the minimum concentration of the proximity label solution required to generate maximum signal.

Once the minimum concentration of the proximity label solution is set, the optimum amount of anti-theophylline antibody required to produce maximum steric hindrance is determined. To make this determination, 100µL of tridentate solution are mixed with 100µL of antibody solution and incubated for about 5 to 15 minutes. (The steady state equilibrium of the antigen:antibody reaction will establish very quickly, in contrast to systems wherein either the antigen or antibody is bound to a solid surface.) A 100µL quantity of the optimized proximity label solution is then added and the entire reaction mixture incubated an additional 5 to 15 minutes. A 700µL aliquot of the substrate solution is finally added and the rate of NADH production monitored using the fluorometer described above.

The assay is repeated with increasing concentrations (decreasing dilutions) of antibody solution until

the rate of NADH production reaches a minimum point, whereupon the addition of increasing concentrations of antibody solution fails to further lower the rate. This is the minimum concentration of antibody solution required to generate maximum steric hindrance.

Assay for Analyte

A 100μL aliquot of a patient's test sample is initially combined with a 100μL aliquot of the tridentate solution. This combined solution is then incubated with 100μL of the optimized antibody solution for a period of approximately 5 to 15 minutes. A 100μL aliquot of the optimized proximity label solution is then added to the incubated mixture, and the combined solution further incubated for an additional 5 to 15 minutes. At this point, 600μL of the substrate solution is added and the rate of NADH formation monitored using a suitable fluorometer.

The same procedure is then repeated for various dilutions of a theophylline or theophylline-amine standard, from which a standard curve can be obtained. The concentration of theophylline or theophylline-amine in the sample can be interpolated from the standard curve.

Example 7

Energy Transfer Using Universal Tridentate

The same biotin-theophylline-DNP tridentate can also be used to perform a competitively modulated energy transfer assay. For example, an energy donor, such as the chemiluminescent molecule isoluminol, is attached to either avidin or anti-DNP antibody. An energy acceptor,

such as fluorescein isothiocyanate, is attached to the other specific binding partner. Free theophylline or theophylline-amine, contributed by a test sample or calibration standard, modulates energy transfer by diverting anti-theophylline antibody away from the second (modulating) member of the tridentate.

Preparation of Isoluminol-Avidin Conjugate

An excess of an isothiocyanate derivative of aminobutylethylamino-isoluminol is dissolved in DMF. An avidin solution is then made by suspending avidin in a 0.1M sodium carbonate/sodium bicarbonate buffer at pH 9.5. The isoluminol-containing DMF solution is then added to the avidin solution and incubated at about 4°C for about 12 hours. The excess isoluminol label can then be removed by extensive dialysis, followed by gel filtration using a Sephadex® G-50 column.

Preparation of Fluorescein-Anti-DNP Antibody Conjugate

Fluorescein-labeled antibody can be prepared in a similar manner. An excess of fluorescein isothiocyanate is dissolved in p-dioxane. An antibody solution is then made by suspending rabbit anti-DNP antibody in a 0.1M sodium carbonate/sodium bicarbonate buffer at pH 9.5. The fluorescein-containing p-dioxane solution is then added to the antibody solution and incubated at about 4°C for about 12 hours. The excess fluorescein label can then be removed by extensive dialysis followed by gel filtration through a Sephadex® G-50 column.

Determination of Optimum Reagent Concentrations for Energy Transfer

The following reagents are used:

Incubation buffer: 50mM phosphate buffer, pH 7.4.

Tridentate solution: tridentate conjugate dissolved in incubation buffer to a concentration equivalent to about 1μg/mL theophylline.

Proximity label solution: equimolar quantities of isoluminol-avidin and fluorescein-anti-DNP antibody suspended in incubation buffer. Various dilutions are prepared.

Antibody solution: anti-theophylline antibody suspended in incubation buffer. Various dilutions are prepared.

Chemiluminescent triggering reagent: 5μM microperoxidase (Sigma Chemicals) in 100mM barbitone buffer, pH 9, 0.01% BSA, and 0.175M H_2O_2.

The maximum attainable chemiluminescence energy transfer at the given concentration of tridentate conjugate is first assessed.

Initially, 100μL of the tridentate solution and 100μL of the undiluted proximity label solution are incubated at room temperature for about 5 to 15 minutes. An aliquot of this mixture is then introduced into a luminometer having two band pass filters of 460nm (fluorescein excitation wavelength) and 525nm (fluorescein emission wavelength) in front of two photomultipliers. An

adequate amount of chemiluminescent triggering reagent is then added to induce light emission by the isoluminol molecules present. The isoluminol molecules emit light at about 460nm. Where the isoluminol is in close proximity with the fluorescein label, fluorescein will absorb the emitted light at 460nm with the concommitant emission of fluorescent light energy at 525nm. The ratio of light levels, 525nm/460nm increases with increasing energy transfer. These measurements are repeated using increasing dilutions of proximity label solution until the ratio of light levels begins to decrease. This establishes the minimum concentration of proximity label solution required to generate maximum signal.

Once the minimum concentration of the proximity label solution is established, the optimum amount of anti-theophylline antibody required to produce maximum steric hindrance is determined. To make this determination, anti-theophylline antibody is added to the incubation mixture prior to addition of the chemiluminescent triggering reagent. Increasing amounts of anti-theophylline antibody are added in subsequent determinations until a point is reached whereupon the addition of increasing concentrations of antibody solution fails to further lower the measurable energy transfer. This is the minimum concentration of antibody solution required to generate maximum steric hindrance.

Assay for Analyte

A 100µL aliquot of a patient's test sample is initially incubated with a 100µL aliquot of the optimized antibody solution. 100µL aliquots of the tridentate solution and proximity label solution are then added and the combined solution further incubated for an additional 5 to 15 minutes. An aliquot of this combined solution is

then introduced into a luminometer and an adequate amount of chemiluminescent triggering reagent added prior to signal being measured.

The same procedure is then repeated for various dilutions of a theophylline or theophylline-amine standard, from which a standard curve can be obtained. The concentration of theophylline or theophylline-amine in the sample can be interpolated from the standard curve.

Example 7

Energy Transfer Where One of Tridentate Members is a Proximity Label

The same general procedure set forth in Example 6 may be followed where a tridentate having a proximity label as one of the tridentate members is employed.

For example, the tridentate shown in Fig. 10A may be employed. In this instance, the proximity label solution contains only the fluorescein-anti-DNP antibody. In all other respects, the same optimization and assay procedures set forth in Example 6 are followed.

Example 8

Energy Transfer Where One of Tridentate Members is a Solid Support

The same general procedure set forth in Example 6 may also be followed where a tridentate having, as one of its members, a macromolecule conjugated to a plurality of proximity labels is employed. The macromolecule may be a solid support as shown in Fig. 11.

Where the tridentate shown in Fig. 11 is employed, the proximity label solution again contains only the fluorescein-anti-DNP antibody. In all other respects, the same optimization and assay procedures set forth in Example 6 are followed, with the exception that incubation times may have to be extended to account for the slower kinetics of a system utilizing a solid support.

Example 9

Attachment of Biotion Label to Glycosylated Protein

The boronic acid-azide-biotin tridentate shown in Fig. 9 may be used to attach a biotin label to a glycosylated protein at a designated site on the protein; i.e., at the sugar moiety. This procedure is particularly useful in biotinylating an antibody, enzyme, or antigen.

Targeted Binding of Guiding Member

The following reagents are used:

Glycosylated protein solution: any glycosylated protein solution, such as antibody solutions or ascites fluid, antigen solutions, and enzyme preparations, may be used. Many of these solutions are commercially available.

Buffer solution: 50mM N-methylmorpholinium chloride, pH 7.2, 100mM magnesium chloride.

Tridentate solution: The tridentate shown in Fig. 9 is dissolved in 10% (w/v) NaOH to deprotect the boronic acid moiety (guiding member) of the tridentate.

The glycosylated protein solution is dialyzed in the buffer solution and set aside. The tridentate solution is then diluted with the same buffer solution. An aliquot of the diluted tridentate solution, representing about a 10 to 100-fold molar excess of the tridentate relative to the total glycosylated protein in the glycosylated protein solution, is then removed. This aliquot is added to the dialyzed glycosylated protein solution, and the entire mixture is incubated at room temperature for about 2 hours in the dark.

Following incubation, the reaction mixture may be chromatographed in the dark on a Sephadex® G-50 column, with the protein fraction being isolated. The protein fraction will contain the boronate complex (bound guiding member).

Attachment of Reactive Member

The isolated product may then be irradiated with a suitable ultraviolet light source, such as a Mazda 125W MB/V pearl glass lamp. The irradiation takes place at 0°C from a distance of about 5 to about 20cm from the illuminator. In order to insure complete conversion of the azide to nitrene, the irradiation may be continued for several hours. Photocoupling of the tridentate to the glycosylated protein takes place almost immediately upon conversion of the azide residue to nitrene. Following the photocoupling reaction, the reaction mixture can be dialyzed in almost any standard buffer solution, using standard dialysis techniques, to eliminate any photochemically uncoupled tridentate.

Still other types of tridentate conjugates and methods of use thereof are contemplated as being within the scope of this invention and will be apparent to those

skilled in the art. As this invention may be embodied in several forms, without departing from the essential spirit thereof, the invention is intended to be defined by the appended claims as opposed to the foregoing description.

What is claimed is:

1. A tridentate conjugate having three tridentate members attached together through an appropriate spacer moiety wherein at least two of said tridentate members are small molecules.

2. The tridentate conjugate of claim 1 wherein at least one of said tridentate members is a small molecule ligand.

3. The tridentate conjugate of claim 2 wherein said spacer moiety is selected such that the binding of a macromolecular specific binding partner to a small molecule ligand member sterically inhibits the binding of a different macromolecule to at least one other tridentate member.

4. The tridentate conjugate of claim 3 wherein said small molecule ligand member is identical or analogous to an analyte of interest.

5. The tridentate conjugate of claim 4 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins.

6. The tridentate conjugate of claim 5 wherein said analyte is a hapten with a molecular weight between about 100 and 1500 Daltons.

7. The tridentate conjugate of claim 1 wherein a first tridentate member and a second tridentate member are capable of binding to the same macromolecule.

8. The tridentate conjugate of claim 9 wherein said first tridentate member is capable of seeking out and binding to a specific location or locations on said macromolecule.

9. The tridentate conjugate of claim 8 wherein said second tridentate member is capable of permanently binding to said macromolecule in the vicinity of said location to which said first tridentate member binds.

10. The tridentate conjugate of claim 9 wherein a third tridentate member is selected from the group consisting of labels, tracers, reporter groups, and solid supports.

11. The tridentate conjugate of claim 10 wherein said first tridentate member is a chemically reactive group.

12. The tridentate conjugate of claim 11 wherein said first tridentate member is a phenyl boronic acid residue.

13. The tridentate conjugate of claim 10 wherein said first tridentate member is a small molecule ligand.

14. The tridentate conjugate of claim 10 wherein said second tridentate member is a chemically reactive group.

15. The tridentate conjugate of claim 14 wherein said second tridentate member is an azide residue.

16. The tridentate conjugate of claim 14 wherein said first and second tridentate members are chemically reactive groups.

17. The tridentate conjugate of claim 16 wherein said first tridentate member is a phenyl boronic acid residue and said second tridentate member is an azide residue.

18. The tridentate conjugate of claim 17 wherein said third tridentate member is biotin.

19. The tridentate conjugate of claim 14 wherein said first tridentate member is a small molecule ligand and said second tridentate member is a chemically reactive group.

20. The tridentate conjugate of claim 19 wherein said first tridentate member is a small molecule ligand and said second tridentate member is an azide residue.

21. The tridentate conjugate of claim 1 wherein at least two of said tridentate members are small molecule ligands.

22. The tridentate conjugate of claim 21 wherein said spacer moiety is selected such that the binding of a first macromolecular specific binding partner to a first small molecule ligand member is sterically inhibited by the binding of a second macromolecular specific binding partner to a second small molecule ligand member.

23. The tridentate conjugate of claim 22 wherein said second small molecule ligand member is identical or analogous to an analyte of interest.

24. The tridentate conjugate of claim 23 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucl otides, and vitamins.

25. The tridentate conjugate of claim 24 wherein said analyte is a hapten with a molecular weight between about 100 and 1500 Daltons.

26. The tridentate conjugate of claim 25 wherein said analyte is selected from the group consisting of theophylline, theophylline-amine, digoxin, disopyramide, lidocaine, procainamide, propanolol, quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, drugs of abuse, such as morphine, codeine, and heroin, and their metabolites, DNP, 1-substituted-4-hydroxy-2-nitrobenzene, and 4-substituted-2-nitro-trialkylanilinium salts.

27. The tridentate conjugate of claim 26 wherein said analyte is selected from the group consisting of theophylline and theophylline-amine.

28. The tridentate conjugate of claim 27 wherein said first small molecule ligand member is selected from the group consisting of biotin and DNP.

29. The tridentate conjugate of claim 23 wherein said first macromolecular specific binding partner is conjugated to a first proximity label.

30. The tridentate conjugate of claim 29 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins.

31. The tridentate conjugate of claim 30 wherein

said analyte is a hapten with a molecular weight between about 100 and 1500 Daltons.

32. The tridentate conjugate of claim 31 wherein said analyte is selected from the group consisting of theophylline, theophylline-amine, digoxin, disopyramide, lidocaine, procainamide, propanolol, quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, drugs of abuse, such as morphine, codeine, and heroin, and their metabolites, DNP, 1-substituted-4-hydroxy-2-nitrobenzene, and 4-substituted-2-nitro-trialkylanilinium salts.

33. The tridentate conjugate of claim 32 wherein said analyte is selected from the group consisting of theophylline and theophylline-amine.

34. The tridentate conjugate of claim 33 wherein said first small molecule ligand member is selected from the group consisting of biotin and DNP.

35. The tridentate conjugate of claim 29 wherein one of said tridentate members is a second proximity label.

36. The tridentate conjugate of claim 35 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins.

37. The tridentate conjugate of claim 36 wherein said analyte of interest is a hapten with a molecular weight between about 100 and 1500 Daltons.

38. The tridentate conjugate of claim 37 wherein said analyte is selected from the group consisting of theophylline, theophylline-amine, digoxin, disopyramide, lidocaine, procainamide, propanolol, quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, drugs of abuse, such as morphine, codeine, and heroin, and their metabolites, DNP, 1-substituted-4-hydroxy-2-nitrobenzene, and 4-substituted-2-nitro-trialkylanilinium salts.

39. The tridentate conjugate of claim 38 wherein said analyte is selected from the group consisting of theophylline and theophylline-amine.

40. The tridentate conjugate of claim 39 wherein said first small molecule ligand is selected from the group consisting of biotin and DNP.

41. The tridentate conjugate of claim 29 wherein one of said tridentate members is a macromolecle to which has been conjugated a plurality of second proximity labels.

42. The tridentate conjugate of claim 41 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins.

43. The tridentate conjugate of claim 42 wherein said analyte of interest is a hapten with a molecular weight between about 100 and 1500 Daltons.

44. The tridentate conjugate of claim 43 wherein

said analyte is selected from the group consisting of theophylline, theophylline-amine, digoxin, disopyramide, lidocaine, procainamide, propanolol, quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, drugs of abuse, such as morphine, codeine, and heroin, and their metabolites, DNP, 1-substituted-4-hydroxy-2-nitrobenzene, and 4-substituted-2-nitro-trialkylanilinium salts.

45. The tridentate conjugate of claim 44 wherein said analyte is selected from the group consisting of theophylline and theophylline-amine.

46. The tridentate conjugate of claim 45 wherein said first small molecule ligand member is selected from the group consisting of biotin and DNP.

47. The tridentate conjugate of claim 35 wherein said first and second proximity labels are selected from the group consisting of energy donors and energy acceptors.

48. The tridentate conjugate of claim 47 wherein said energy donors are selected from the group consisting of fluorescent compounds, scintillation dyes, and chemiluminescent compounds.

49. The tridentate conjugate of claim 47 wherein said energy acceptors are selected from the group consisting of fluorescein, rhodamine, fluorescent lanthanide chelates, and fluorescent tin or zinc derivatives of protoporphyrins.

50. The tridentate conjugate of claim 48 wherein said energy donor is isoluminol.

51. The tridentate conjugate of claim 48 wherein said energy donor is acridin ester.

52. The tridentate conjugate of claim 49 wherein said energy acceptor is fluorescein.

53. The tridentate conjugate of claim 49 wherein said energy acceptor is rhodamine.

54. The tridentate conjugate of claim 52 wherein said energy donor is isoluminol and said energy acceptor is fluorescein.

55. The tridentate conjugate of claim 52 wherein said energy donor is acridin ester and said energy acceptor is fluorescein.

56. The tridentate conjugate of claim 53 wherein said energy donor is fluorescein and said energy acceptor is rhodamine.

57. The tridentate conjugate of claim 35 wherein said first and second proximity labels are enzymes.

58. The tridentate conjugate of claim 57 wherein said enzymes are selected from the group consisting of glucose oxidase and peroxidase.

59. The tridentate conjugate of claim 57 wherein said first and second enzymes are selected from the group consisting of hexokinase and glucose-6-phosphate dehydrogenase.

60. The tridentate conjugate of claim 41 wherein said first and second proximity labels are selected from the group consisting of energy donors and energy acceptors.

61. The tridentate conjugate of claim 60 wherein said energy donors are selected from the group consisting of fluorescent compounds, scintillation dyes, and chemiluminescent compounds.

62. The tridentate conjugate of claim 60 wherein said energy acceptors are selected from the group consisting of fluorescein, rhodamine, fluorescent lanthanide chelates, and fluorescent tin or zinc derivatives of protoporphyrins.

63. The tridentate conjugate of claim 61 wherein said energy donor is isoluminol.

64. The tridentate conjugate of claim 61 wherein said energy donor is acridin ester.

65. The tridentate conjugate of claim 62 wherein said energy acceptor is fluorescein.

66. The tridentate conjugate of claim 62 wherein said energy acceptor is rhodamine.

67. The tridentate conjugate of claim 65 wherein said energy donor is isoluminol and said energy acceptor is fluorescein.

68. The tridentate conjugate of claim 65 wherein said energy donor is acridin ester and said energy acceptor is fluorescein.

69. The tridentate conjugate of claim 66 wherein said energy donor is fluorescein and said energy acceptor is rhodamine.

70. The tridentate conjugate of claim 41 wherein said first and second proximity labels are enzymes.

71. The tridentate conjugate of claim 70 wherein said enzymes are selected from the group consisting of glucose oxidase and peroxidase.

72. The tridentate conjugate of claim 70 wherein said first and second proximity labels are selected from the group consisting of hexokinase and glucose-6-phosphate dehydrogenase.

73. The tridentate conjugate of claim 21 wherein a first tridentate member and a second tridentate member are capable of binding to the same macromolecule.

74. The tridentate conjugate of claim 73 wherein said first tridentate member is capable of seeking out and binding to a specific location or locations on said macromolecule.

75. The tridentate conjugate of claim 74 wherein said second tridentate member is capable of permanently binding to said macromolecule in the vicinity of said location to which said first tridentate member binds.

76. The tridentate conjugate of claim 75 wherein a third tridentate member is selected from the group consisting of labels, tracers, reporter groups, and solid supports.

77. The tridentate conjugate of claim 76 wherein

said first tridentate member is a small molecule ligand and said second tridentate member is a chemically reactive group.

78. The tridentate conjugate of claim 77 wherein said first tridentate member is a small molecule ligand and said second tridentate member is an azide residue.

79. The tridentate conjugate of claim 1 wherein all three of said tridentate members are small molecule ligands.

80. The tridentate conjugate of claim 79 wherein said spacer moiety is selected such that the binding of a first macromolecular specific binding partner to a first small molecule ligand member is sterically inhibited by the binding of a second macromolecular specific binding partner to a second small molecule ligand member.

81. The tridentate conjugate of claim 80 wherein said second small molecule ligand member is identical or analogous to an analyte of interest.

82. The tridentate conjugate of claim 81 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins.

83. The tridentate conjugate of claim 82 wherein said analyte is a hapten with a molecular weight between about 100 and 1500 Daltons.

84. The tridentate conjugate of claim 83 wherein said analyte of interest is selected from the group consisting of theophylline, theophylline-amine, digoxin, disopyramide, lidocaine, procainamid , propanolol,

quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, drugs of abuse, such as morphine, codeine, and heroin, and their metabolites, DNP, 1-substituted-4-hydroxy-2-nitrobenzene, and 4-substituted-2-nitro-trialkylanilinium salts.

85. The tridentate conjugate of claim 84 wherein said analyte is selected from the group consisting of theophylline and theophylline-amine.

86. The tridentate conjugate of claim 85 wherein said first small molecule ligand member is selected from the group consisting of biotin and DNP.

87. The tridentate conjugate of claim 80 wherein a third macromolecular specific binding partner is capable of specifically binding to a third small molecule ligand member.

88. The tridentate conjugate of claim 87 wherein said first macromolecular specific binding partner and said third macromolecular specific binding partner are polyvalent.

89. The tridentate conjugate of claim 88 wherein said second small molecule ligand member is identical or analogous to an analyte of interest.

90. The tridentate conjugate of claim 89 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins.

91. The tridentate conjugate of claim 90 wherein said analyte is a hapten with a molecular weight between about 100 and 1500 Daltons.

92. The tridentate conjugate of claim 91 wherein said analyte of interest is selected from the group consisting of theophylline, theophylline-amine, digoxin, disopyramide, lidocaine, procainamide, propanolol, quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, drugs of abuse, such as morphine, codeine, and heroin, and their metabolites, DNP, 1-substituted-4-hydroxy-2-nitrobenzene, and 4-substituted-2-nitro-trialkylanilinium salts.

93. The tridentate conjugate of claim 92 wherein said analyte is selected from the group consisting of theophylline and theophylline-amine.

94. The tridentate conjugate of claim 93 wherein said first and third small molecule ligand members are selected from the group consisting of biotin and DNP.

95. The tridentate conjugate of claim 87 wherein said first macromolecular specific binding partner is conjugated to a first proximity label and said second macromolecular specific binding partner is conjugated to a second proximity label.

96. The tridentate conjugate of claim 95 wherein said first and second proximity labels are selected from the group consisting of energy donors and energy acceptors.

97. The tridentate conjugate of claim 96 wherein said energy donors are selected from the group consisting of fluorescent compounds, scintillation dyes, and chemiluminescent compounds.

98. The tridentate conjugate of claim 96 wherein said energy acceptors are selected from the group consisting of fluorescein, rhodamine, fluorescent lanthanide chelates, and fluorescent tin or zinc derivatives of photoporphyrins.

99. The tridentate conjugate of claim 97 wherein said energy donortor is isoluminol.

100. The tridentate conjugate of claim 97 wherein said energy donor is acridin ester.

101. The tridentate conjugate of claim 98 wherein said energy acceptor is fluorescein.

102. The tridentate conjugate of claim 98 wherein said energy acceptor is rhodamine.

103. The tridentate conjugate of claim 101 wherein said energy donor is isoluminol and said energy acceptor is fluorescein.

104. The tridentate conjugate of claim 101 wherein said energy donor is acridin ester and said energy acceptor is fluorescein.

105. The tridentate conjugate of claim 102 wherein said energy donor is fluorescein and said energy acceptor is rhodamine.

106. The tridentate conjugate of claim 95 wherein said first and second proximity labels are enzymes.

107. The tridentate conjugate of claim 106 wherein said enzymes are selected from the group consisting of glucose oxidase and peroxidase.

108. The tridentate conjugate of claim 106 wherein said enzymes are selected from the group consisting of hexokinase and glucose-6-phosphate dehydrogenase.

109. A competitive immunoassay method comprising:

(a) contacting a test sample, which may or may not contain an analyte of interest with: (1) a limited quantity of macromolecular specific binding partner for said analyte of interest; (2) a tridentate conjugate as described in claim 4; and (3) an excess of a different macromolecule capable of binding to said tridentate in the absence of binding by said macromolecular specific binding partner for said analyte of interest; and,

(b) detecting the amount of said macromolecular specific binding partner for said analyte of interest diverted away from said tridentate to determine the presence of said analyte of interest in said test sample.

110. The method of claim 109 wherein said tridentate conjugate is the tridentate conjugate of claim 23.

111. The method of claim 110 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and

vitamins, and said tridentate conjugate is the tridentate conjugate of claim 24.

112. The method of claim 111 wherein said analyte of interest is a hapten with a molecular weight between about 100 and 1500 Daltons, said tridentate conjugate is the tridentate conjugate of claim 25, and said macromolecular specific binding partner for said analyte of interest is an antibody.

113. A competitive immunoassay method comprising:

(a) contacting a test sample, which may or may not contain an analyte of interest, with: (1) a tridentate conjugate as described in claim 23; (2) an excess of a macromolecular specific binding partner for said first small molecule ligand member of said tridentate conjugate; and, (3) a limited quantity of macromolecular specific binding partner for said analyte of interest; and,

(b) detecting the amount of said macromolecular specific binding partner for said analyte of interest diverted away from said tridentate to determine the presence of said analyte of interest in said test sample.

114. The method of claim 113 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins, and said tridentate conjugate is the tridentate conjugate of claim 24.

115. The method of claim 114 wherein said analyte of interest is a hapten with a molecular weight between about 100 and 1500 Daltons, said tridentate conjugate is

the tridentate conjugate of claim 25, and said macromolecular specific binding partner for said analyte of interest is an antibody.

116. The method of claim 113 wherein said tridentate conjugate is the tridentate conjugate of claim 35.

117. The method of claim 116 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins, and said tridentate conjugate is the tridentate conjugate of claim 36.

118. The method of claim 117 wherein said analyte of interest is a hapten with a molecular weight between about 100 and 1500 Daltons, said tridentate conjugate is the tridentate conjugate of claim 37, and said macromolecular specific binding partner for said analyte of interest is an antibody.

119. The method of claim 113 wherein said tridentate conjugate is the tridentate conjugate of claim 41.

120. The method of claim 119 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins, and said tridentate conjugate is the tridentate conjugate of claim 42.

121. The method of claim 120 wherein said analyte of interest is a hapten with a molecular weight between about 100 and 1500 Daltons, said tridentate conjugate is the tridentate conjugate of claim 43, and said

macromolecular specific binding partner for said analyte of interest is an antibody.

122. The method of claim 113 wherein said tridentate conjugate is the tridentate conjugate of claim 87 and said test sample is further contacted with an excess of a third macromolecular specific binding partner for said third small molecule ligand member of said tridentate conjugate.

123. The method of claim 122 wherein said first macromolecular specific binding partner and said third macrololecular specific binding partner are polyvalent and said tridentate conjugate is the tridentate conjugate of claim 88.

124. The method of claim 123 wherein said analyte of interest is selected from the group consisting of haptens, hormones, polypeptides, oligonucleotides, and vitamins, and said tridentate conjugate is the tridentate conjugate of claim 90.

125. The method of claim 124 wherein said analyte of interest is a hapten with a molecular weight between about 100 and 1500 Daltons, said tridentate conjugate is the tridentate conjugate of claim 91, and said macromolecular specific binding partner for said analyte of interest is an antibody.

126. The method of claim 125 wherein said analyte of interest is selected from the group consisting of theophylline, theophylline-amine, digoxin, disopyramide, lidocaine, procainamide, propanolol, quinidine, amykamycin, chloramphenicol, gentamicin, kanamycin, netilmycin, tobramycin, tricyclic antidepressants, ethosuximide, phenobarbital, phenytoin, primidone,

valproic acid, acetaminophen, acetylsalicylic acid, methotrexate, drugs of abuse, such as morphin , codeine, and heroin, and their metabolites, DNP, 1-substituted-4-hydroxy-2-nitrobenzene, and 4-substituted-2-nitro-trialkylanilinium salts, and said tridentate conjugate is the tridentate conjugate of claim 92.

127. The method of claim 126 wherein said analyte of interest is selected from the group consisting of theophylline and theophylline-amine, said tridentate conjugate is the tridentate conjugate of claim 93, and said first and third macromolecular specific binding partners are selected from the group consisting of biotin and DNP.

128. The method of claim 122 wherein said tridentate conjugate is the tridentate conjugate of claim 95.

129. A method of attaching an intended label selected from the group consisting of labels, tracers, reporter groups, and solid supports to a glycosylated protein comprising:

(a) contacting said glycosylated protein with the tridentate conjugate of claim 17 to form a mixture; and,

(b) subsequently exposing the mixture to ultraviolet light.

130. The method of claim 129 wherein said intended label is biotin.

131. A method of making a tridentate conjugate wherein three tridentate members are individually

attached to a starting spacer moiety having three distinct organic functional groups which are capable of being individually derivatized.

132. The method of claim 131 wherein said starting spacer moiety has at least two different functional groups.

133. The method of claim 132 wherein two of said functinal groups are identical.

134. The method of claim 133 wherein one of said identical functional groups is protected by a protecting group.

135. The method of claim 134 wherein said starting spacer moiety is a protected amino acid.

136. The method of claim 135 wherein said protecting group is selected from the group consisting of benzyl esters, tertiary-butyl esters, carbobenzoxy esters, benzoyloxy-carbonylphthalyl, and 9-fluorenyl-methyloxy-carbonyl.

137. The method of claim 136 wherein said starting spacer moiety is carbobenzoxylysine.

138. The method of claim 134 wherein one of said identical functional groups is self-protected.

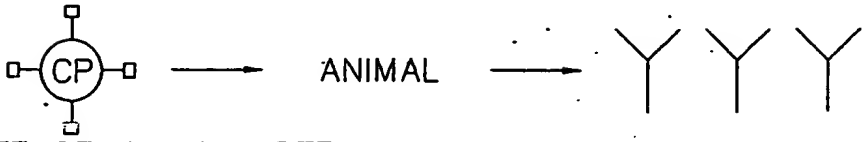
139. The method of claim 138 wherein said starting spacer moiety is a cyclic acid anhydride.

140. The method of claim 139 wherein said starting spacer moiety is selected from the group consisting of pyroglutamic acid and S-acetyl-mercaptosuccinic acid.

141. The method of claim 131 wherein said starting spacer moiety has three different functional groups.

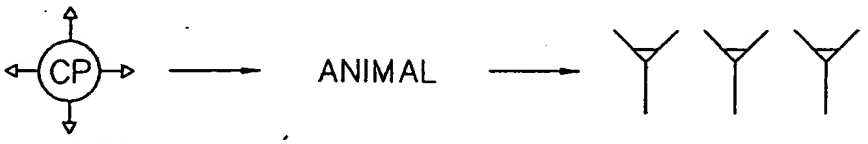
142. The method of claim 141 wherein said starting spacer moiety is a mercapto amino acid.

A. PRODUCTION OF ANTISERUM TO HAPTEN



CONJUGATE OF FIRST HAPTEN (ANALYTE)

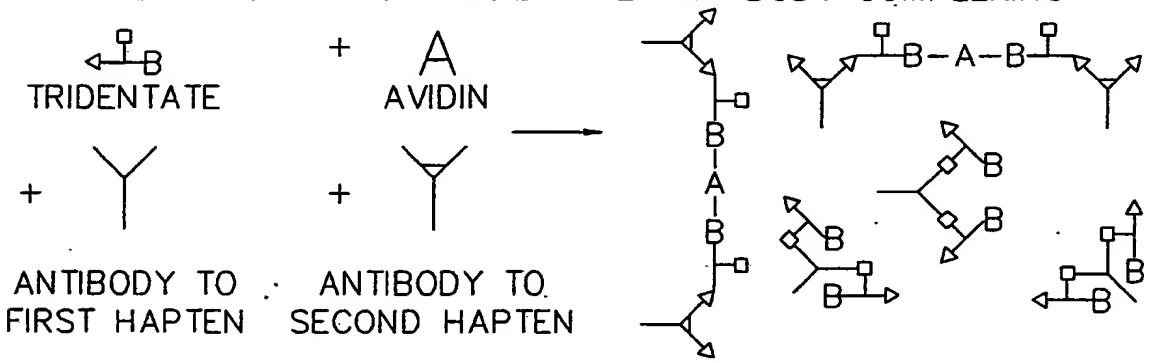
ANTIBODIES TO FIRST HAPTEN



CONJUGATE OF SECOND HAPTEN

ANTIBODY TO SECOND HAPTEN

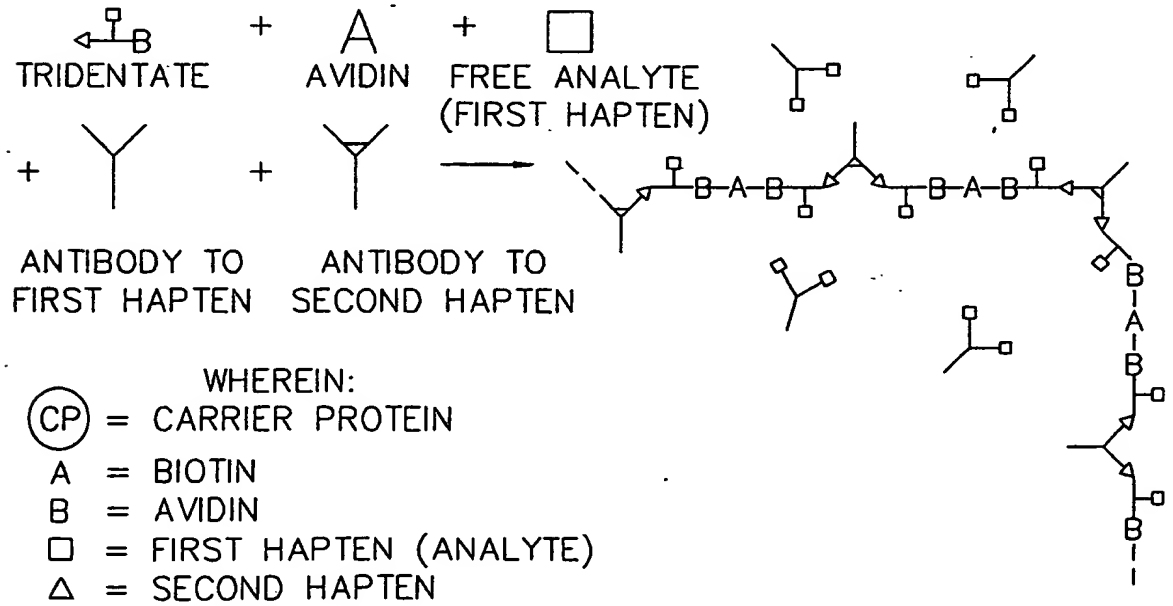
B. INHIBITION OF TRIDENTATE: ANTIBODY COMPLEXING



ANTIBODY TO FIRST HAPTEN

ANTIBODY TO SECOND HAPTEN

C. TRIDENTATE: ANTIBODY COMPLEXING IN PRESENCE OF FREE ANALYTE (FIRST HAPTEN)



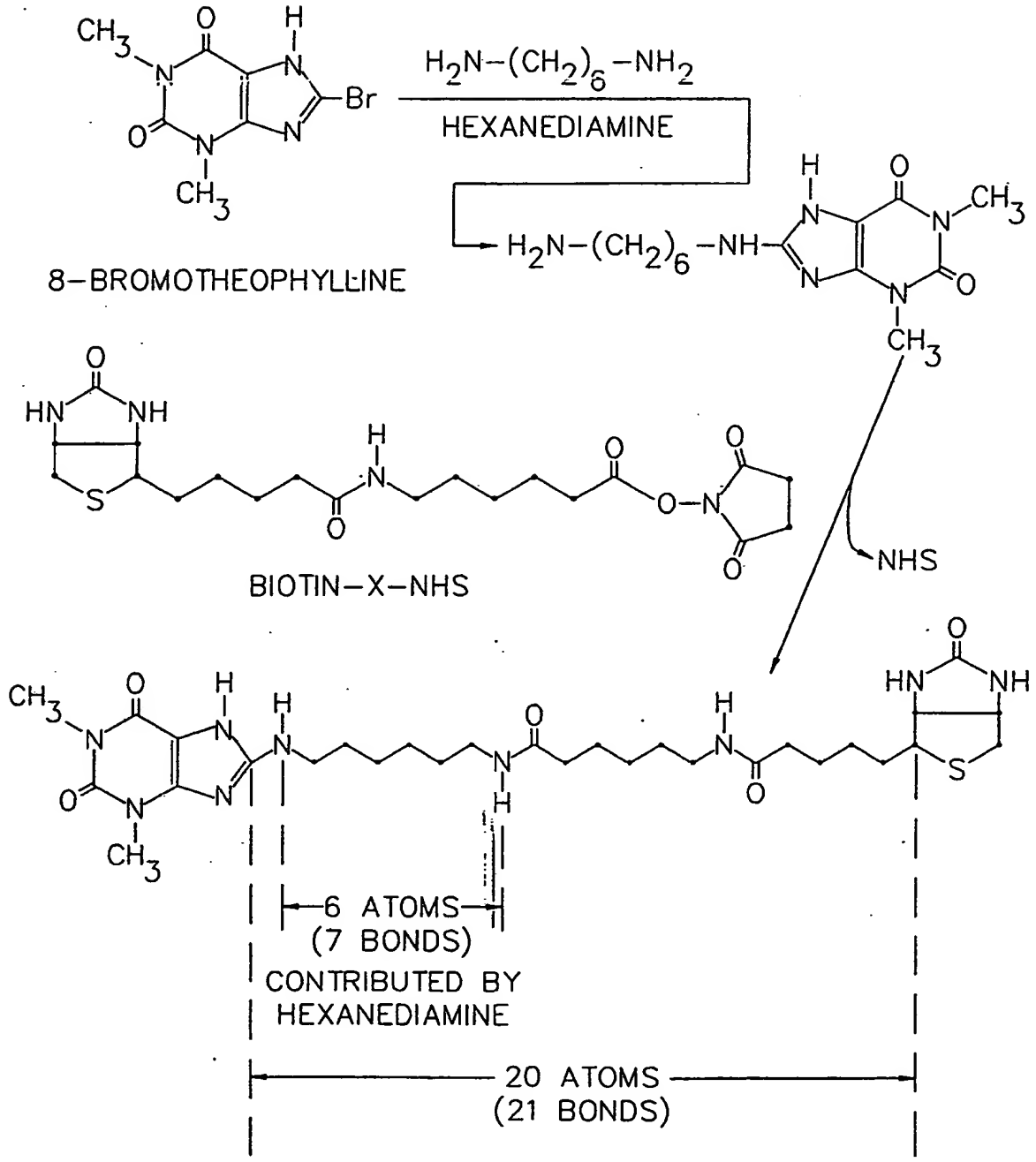
ANTIBODY TO FIRST HAPTEN

ANTIBODY TO SECOND HAPTEN

WHEREIN:

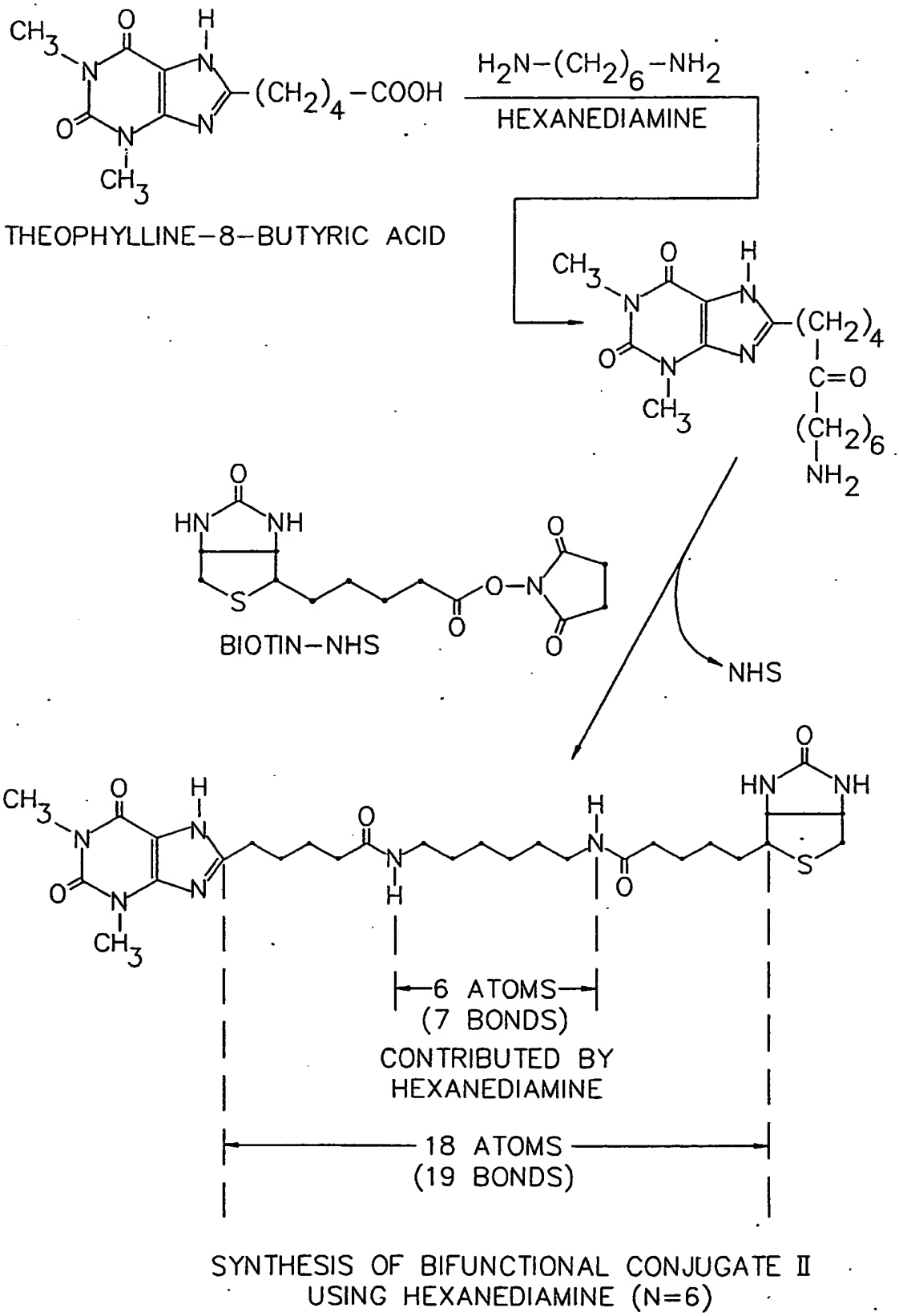
(CP) = CARRIER PROTEIN
A = BIOTIN
B = AVIDIN
□ = FIRST HAPTEN (ANALYTE)
△ = SECOND HAPTEN
Y = ANTIBODY TO FIRST HAPTEN
Y = ANTIBODY TO SECOND HAPTEN

FIG. 1



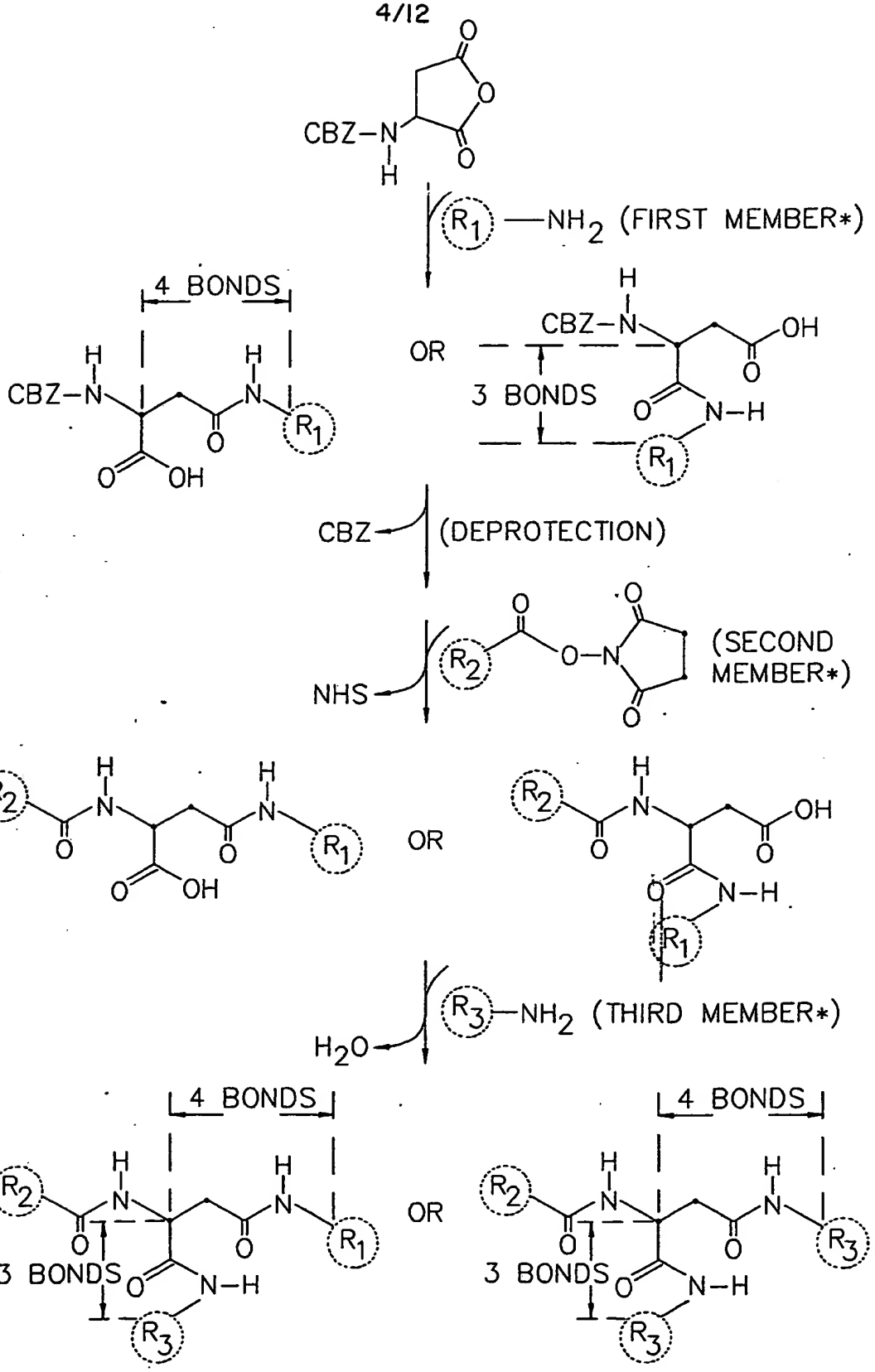
SYNTHESIS OF BIFUNCTIONAL CONJUGATE I
USING HEXANEDIAMINE (N=6)

FIG. 2



SYNTHESIS OF BIFUNCTIONAL CONJUGATE II
USING HEXANEDIAMINE (N=6)

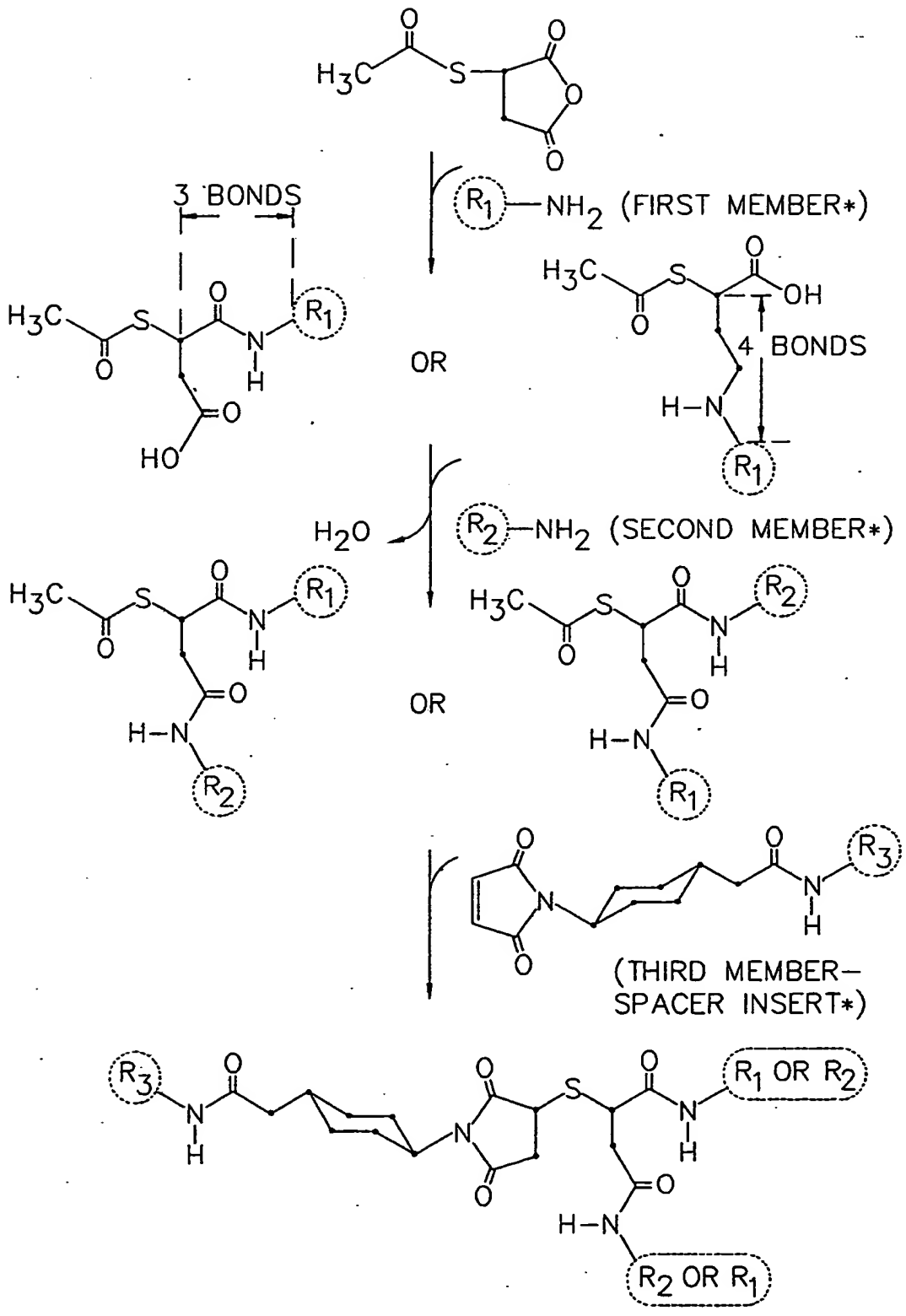
FIG. 3



SYNTHESIS OF TRIDENTATE FROM PYROGLUTAMIC ACID

* MAY INCLUDE ADDITIONAL SPACER INSERT

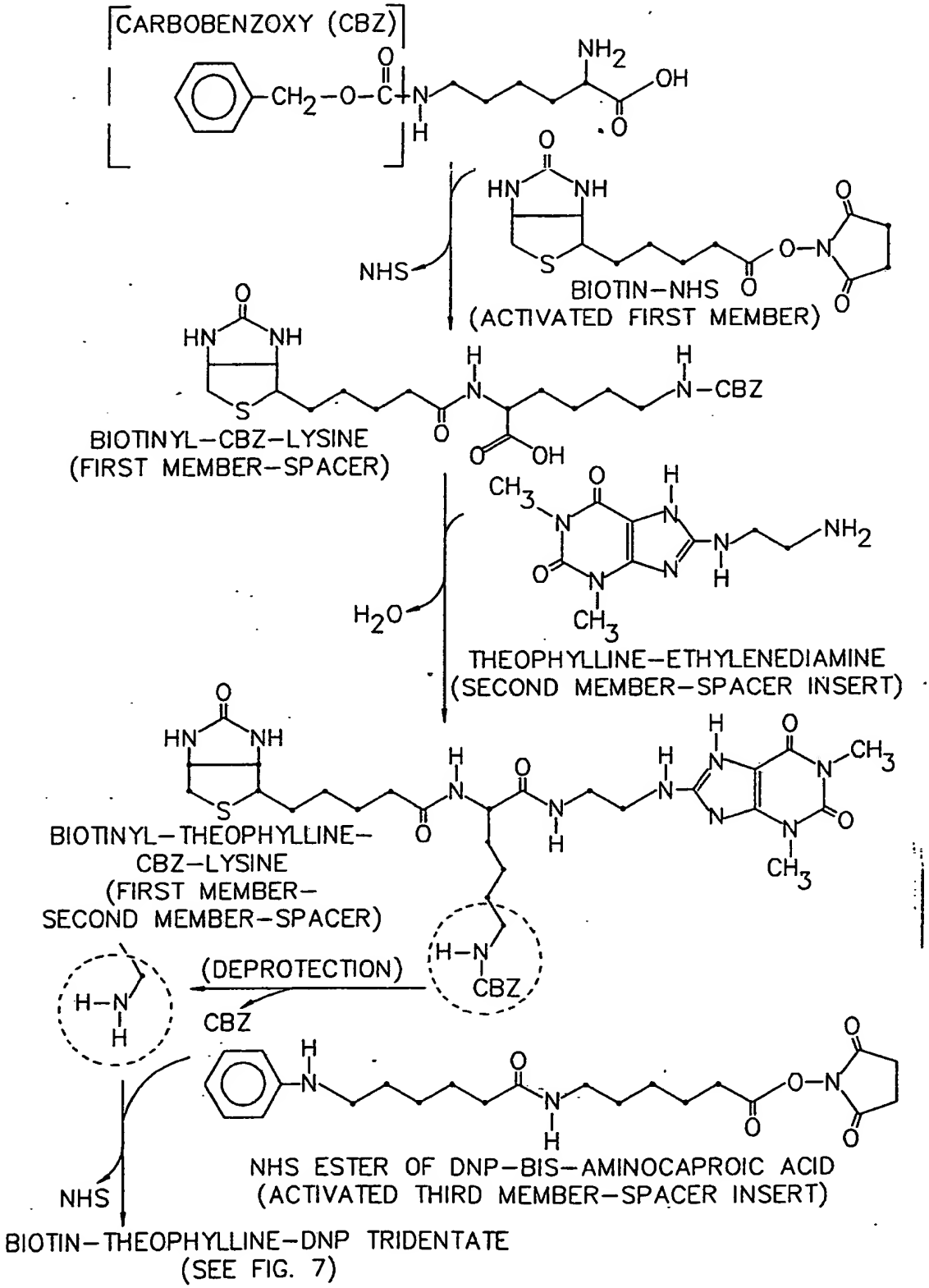
FIG. 4



SYNTHESIS OF TRIDENTATE FROM
S—ACETYL—MERCAPTOSUCCINIC ANHYDRIDE

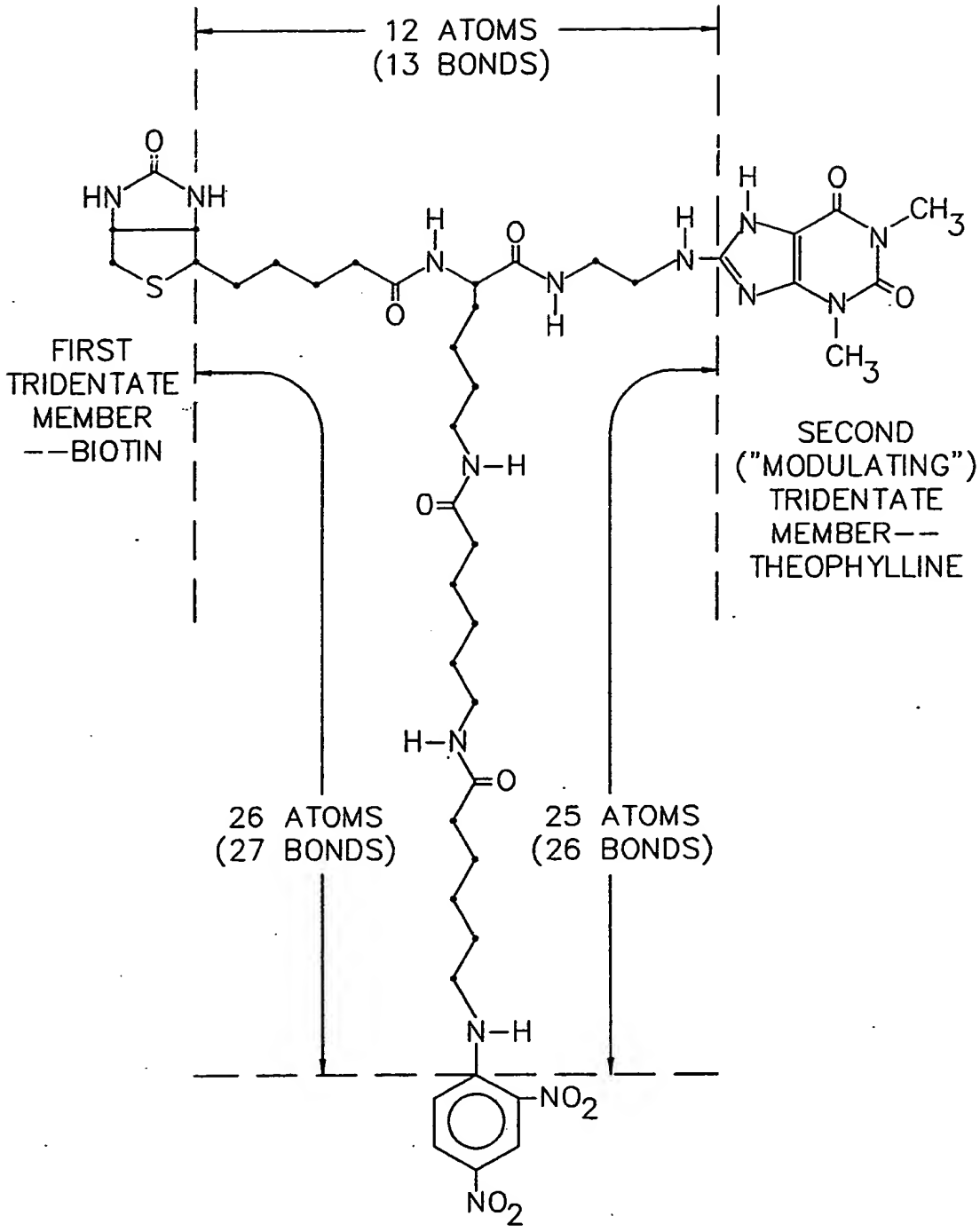
* MAY INCLUDE
ADDITIONAL SPACER INSERT

FIG. 5



SYNTHESIS OF STERIC HINDRANCE TRIDENTATE
FROM CARBOBENZOXYLYSINE

FIG. 6



BIOTIN-THEOPHYLLINE-DNP TRIDENTATE
(STERIC HINDRANCE ENBODIMENT)

FIG. 7

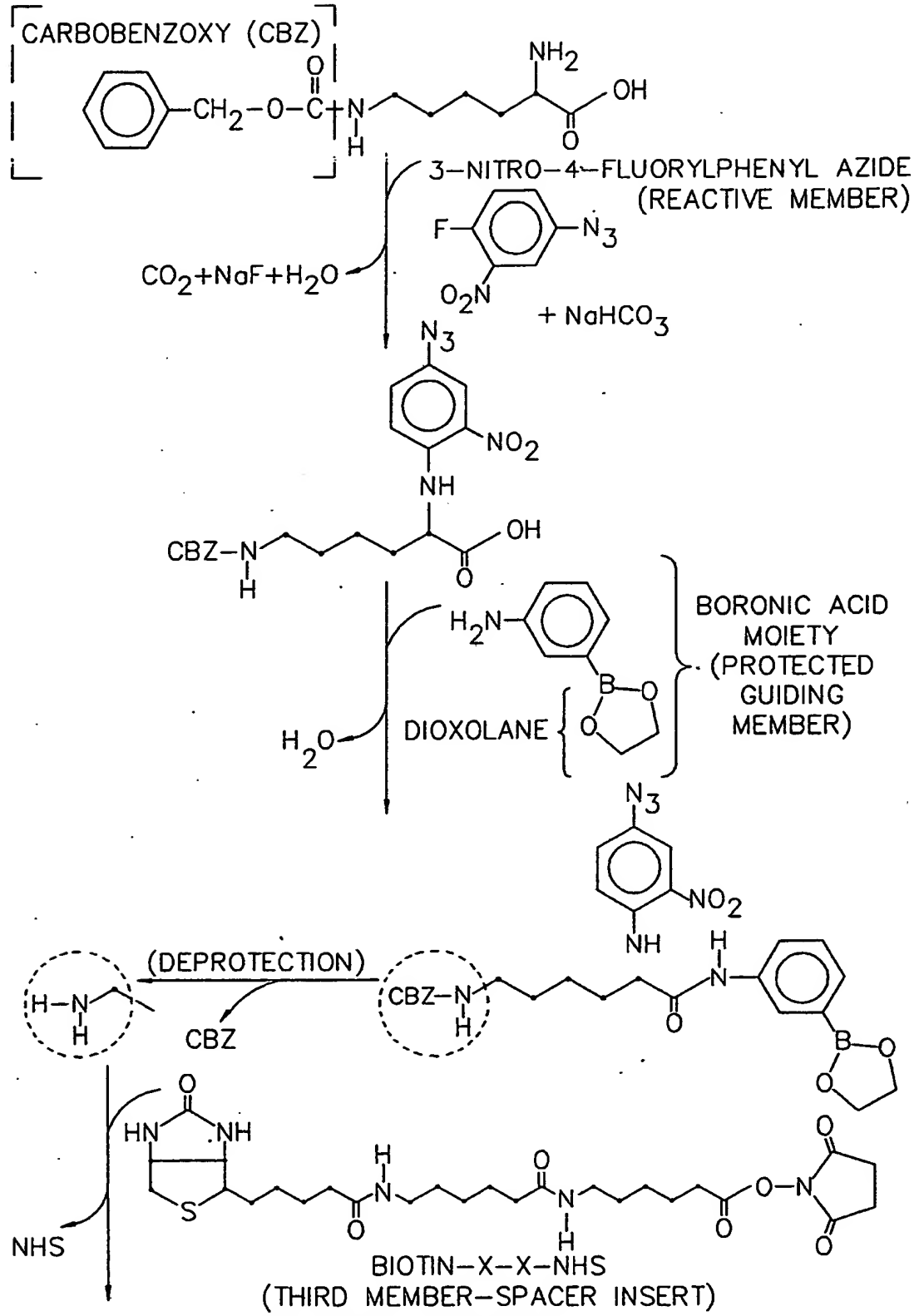


FIG. 8 SYTHESIS OF TARGETED LABELING TRIDENTATE FROM CARBOBENZOXYLYSINE

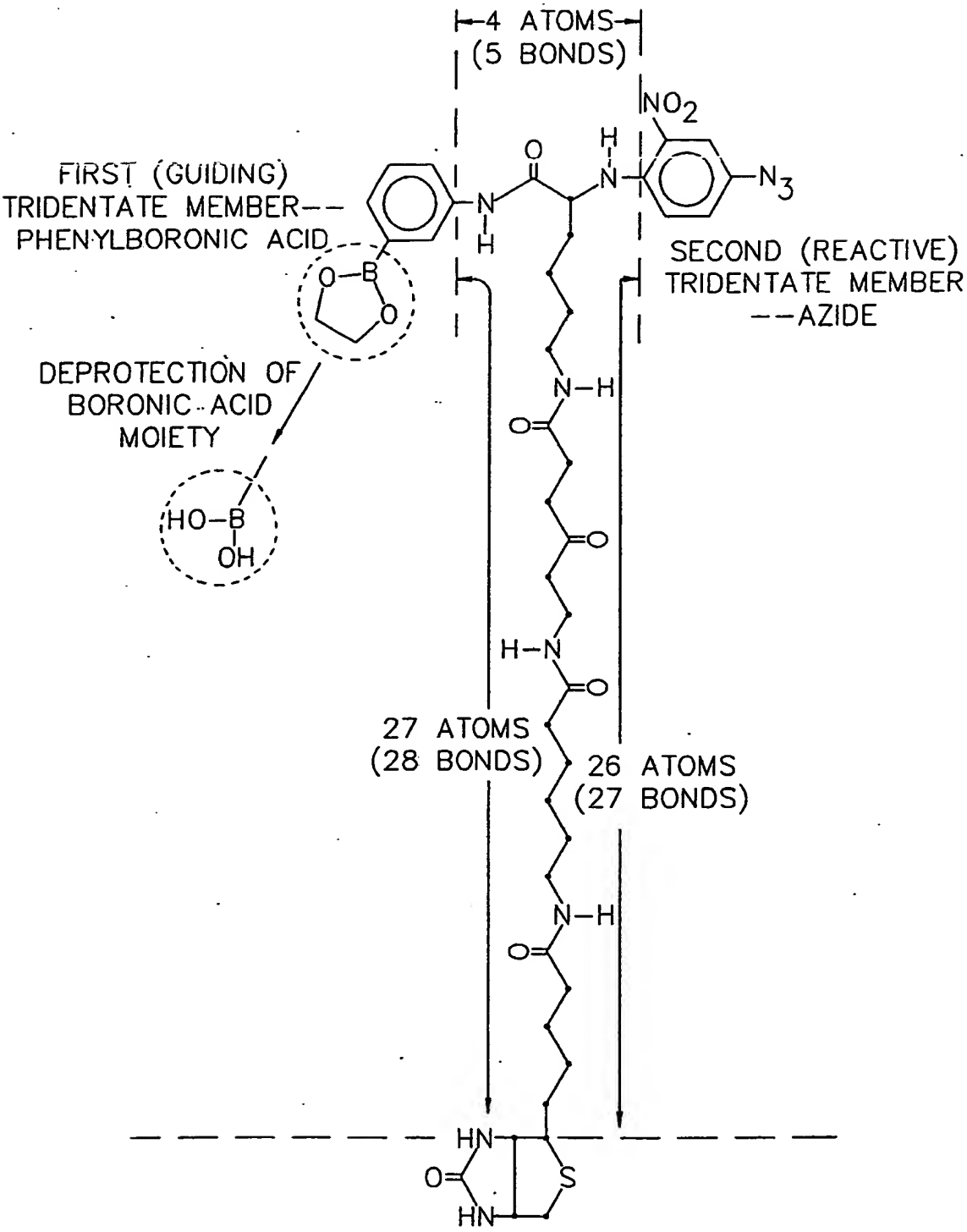


FIG. 9

BORONIC ACID–AZIDE–BIOTIN TRIDENTATE
(TARGETED LABELING EMBODIMENT)

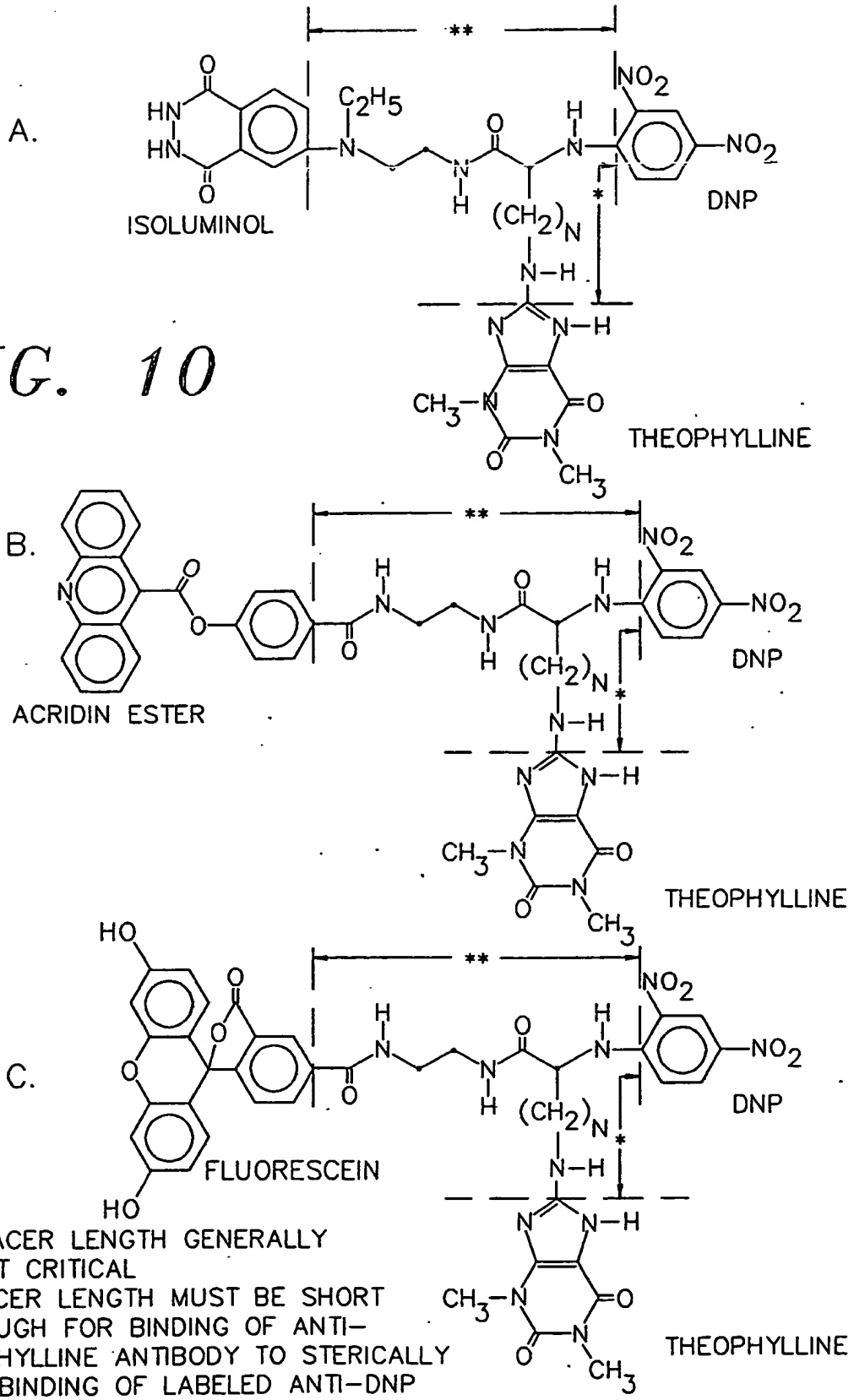
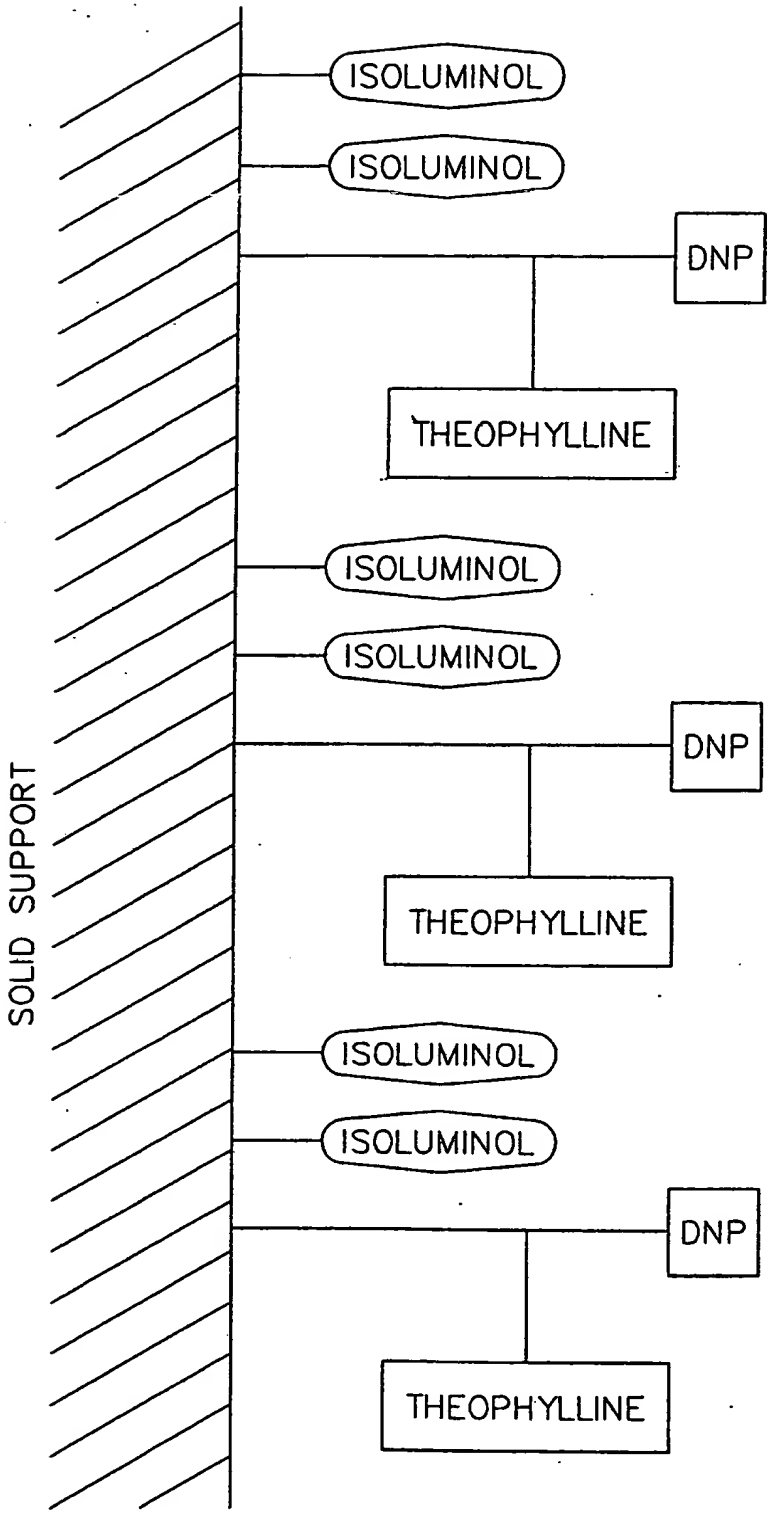


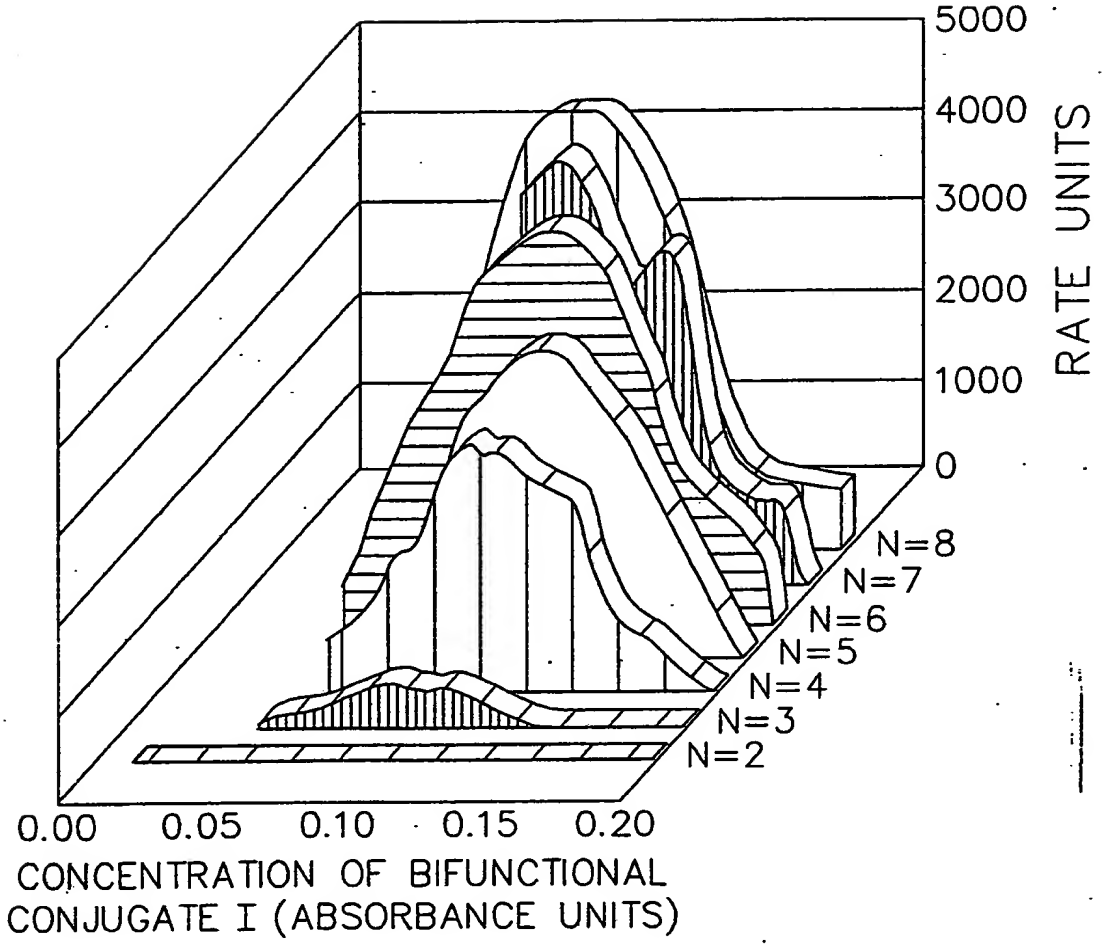
FIG. 10

TRIDENTATES EMPLOYING PROXIMITY LABEL AS ONE OF TRIDENTATE MEMBERS



TRIDENTATE EMPLOYING SOLID SUPPORT AS ONE OF TRIDENTATE MEMBERS

FIG. 11



EFFECT ON BINDING ABILITY OF SPACER LENGTH
BETWEEN TWO TRIDENTATE MEMBERS

FIG. 12